Interaction of σ^{70} with *Escherichia coli* RNA polymerase core enzyme studied by surface plasmon resonance

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Abstract The interaction between the core form of bacterial RNA polymerases and σ factors is essential for specific promoter recognition, and for coordinating the expression of different sets of genes in response to varying cellular needs. The interaction between *Escherichia coli* core RNA polymerase and σ^{70} has been investigated by surface plasmon resonance. The His-tagged form of σ^{70} factor was immobilised on a Ni^{2+}–NTA chip for monitoring its interaction with core polymerase. The binding constant for the interaction was found to be 1.9×10^{-7} M, and the dissociation rate constant for release of σ from core, in the absence of DNA or transcription, was 4×10^{-3} s^{-1}, corresponding to a half-life of about 200 s.

Key words: Surface plasmon resonance; RNA polymerase; σ factors; *Escherichia coli*

1. Introduction

The DNA-dependent RNA polymerase of *Escherichia coli* (EC 2.7.7.6) is a multisubunit enzyme that occurs in two forms, the core enzyme, $\alpha_2\beta\beta'$ (M_r = 378 kDa) which associates reversibly with a σ factor to form the holoenzyme $\alpha_2\beta\beta'\sigma$ (M_r = 448 kDa) [1,2]. The holoenzyme binds DNA at promoter sites and initiates specific transcription in the absence or presence of ancillary regulatory proteins depending on the promoter [3]. Once transcription elongation has begun, the σ factor dissociates from the holoenzyme, and the core polymerase continues to translocate along DNA synthesising RNA. The released σ factor can subsequently bind to another core polymerase to initiate a new round of transcription [4]. *Escherichia coli* contains seven different σ factors, which associate with core to direct transcription to different subsets of genes [5]. The reversible binding of alternative σ factors allows RNA polymerase to recognise different groups of promoters and coordinate the expression of different sets of genes in response to varying cellular needs. Promoter selectivity is therefore dependent on competition between these σ factors, which itself depends on the relative abundance of the various σ factors, their affinities for the core enzyme, and the presence of specific anti-σ factors [6]. Bacterial σ factors fall into two classes; the larger family comprises factors with close structural and functional similarities to the major σ^{70} factor of *E. coli*, which is responsible for transcribing the major 'housekeeping' genes. σ^N is the only known representative that does not belong to this class; its amino acid sequence bears little resemblance to that of σ^{70} and there are important mechanistic differences in the action of RNA polymerase when associated with σ^{70} or σ^N [7]. However, recent studies using tethered iron chelate (Fe-BABE) derivatives indicate that, despite the lack of sequence similarity and functional differences between σ^N and the σ^{70} family, many of the sites of interaction on the core RNA polymerase are similar, implying that there is a common use of at least some interacting regions on the core enzyme [8].

We have recently shown, using σ factors labelled with tryptophan analogues, that the minor σ^N factor associates quite tightly with the core RNA polymerase, and that the kinetic pathway of binding to core is a two-step process in which a fast bimolecular association process is followed by a relatively slow isomerisation step [9]. The binding and kinetic parameters of the σ^N–core polymerase interaction are similar to those previously established for the σ^{70}–core interaction using an *N*-pyrene maleimide derivative of the σ^{70} factor as a fluorescent label to follow binding to core [10].

The competition between the different σ factors for core has not been investigated quantitatively. Of the various approaches that can be used to study protein–protein interactions and competitive binding of different factors to what appears to be a common region on the polymerase, biosensor technology based on surface plasmon resonance (SPR) has many advantages: binding can be monitored in real time; competition experiments with one or more competitive ligands can be carried out readily; and the dynamics of binding can also be studied [11]. To establish the viability of this approach with the present system, and to provide quantitative information on the prototype interaction of core RNA polymerase with σ^{70}, we have investigated the binding of core to a His-tagged form of σ^{70} immobilised to a Ni^{2+}–NTA sensor chip specifically designed for use with His-tagged proteins. Fenton et al. have used a similar approach to monitor the stability, as reflected by dissociation half-lives, of the σ–core interaction as part of a mutant analysis of σ^{70} [12].

2. Materials and methods

2.1. Protein expression and purification

2.1.1. His-tagged σ^{70}. *E. coli* strains BL21 (λDE3) and B834/pLysS (chloramphenicol^r) were transformed with pGEMHσ^{70} (Amp^r) for expression of the N-terminally His-tagged σ^{70}. A 10 cm^3 overnight culture in LB (+100 μg/cm^3 Amp and 25 μg/cm^3 chloramphenicol) was used to inoculate 500 cm^3 of pre-warmed LB+antibiotics. The culture was allowed to grow at 37°C until the OD_{600} was about 0.7, when protein expression was induced with 1 mM IPTG

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supplemented with fresh ampicillin (100 μg/cm^3). After 6–9 h, cells were harvested by centrifugation (5000 rpm for 10 min), resuspended in lysis buffer (50 mM Tris–HCl pH 8.0; 1 mM EDTA; protease inhibitor cocktail tablets, Complete™ Mini, Boehringer) and lysed by treatment at 0°C with lysozyme (3 mg) for 20 min, deoxycholate (20 mg) for 20 min, and sonication (6×10 s pulses, with 1 min pauses in between). The lysate was centrifuged (13 000 rpm for 30 min), and the pellet was resuspended in 20 cm^3 wash buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.1 M NaCl, 0.5% Triton X-100, phenylmethylsulphonyl fluoride 1 mM) for 1 h at 4°C. After centrifugation (15 000 for 15 min) the precipitate was solubilised in 40 cm^3 denaturation buffer (6 M guanidinium–HCl, 10 mM Tris–HCl pH 8.0, 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT)), and renatured by dialysis (4°C, overnight) against the same buffer without the guanidinium–HCl.

Renatured His-tagged σ^{70} protein was purified by FPLC (Pharmacia) using a Ni-chelating column (5 cm^3 HiTRAP chelating, Pharmacia) after dialysis into FPLC buffer A (0.02 M Na_2PO_4 pH 7.2, 0.5 M NaCl). His-tagged σ^{70} bound to the Ni^{2+}-chelating column was eluted with increasing imidazole concentration using a linear gradient of buffer A and buffer B (0.02 M Na_2PO_4 pH 7.2, 0.5 M NaCl, 1 M imidazole) and fractions were analysed by SDS–PAGE. Fractions containing σ^{70} were pooled, dialysed into buffer Uno-A (10 mM Tris–HCl, pH 8.0, 5% glycerol, 1 mM EDTA, 0.1 mM DTT, 50 mM NaCl), and applied to an UnoQ-6R column (Bio-Rad) and eluted with a linear gradient of buffer Uno-A and buffer Uno-B (buffer Uno-A with 1 M NaCl). Fractions containing pure His-tagged σ^{70}, as analysed by SDS–PAGE, were combined and dialysed into buffer Uno-A containing 50% glycerol and stored at −80°C. Protein purity was shown by SDS–PAGE to be greater than 98%. Protein concentrations were determined spectrophotometrically using an extinction coefficient at 280 nm of 0.60 for a 1 mg/cm^3 solution and a molecular mass of 70.2 kDa [9]. The preparation of His-tagged σ^{70} was characterised by equilibrium and sedimentation velocity analytical ultracentrifugation. It was shown that, at concentrations below 5 μM, protein existed as monomer, but at higher concentrations some aggregation occurred. This appears to be a feature of the His tag, since untagged σ did not aggregate under these conditions. The concentrations of σ^{70} loaded onto the SPR chip was a factor of 10 lower than this, so the SPR results relate to conditions where the protein was completely monomeric.

2.1.2. Core RNA polymerase. Mixed holo and core polymerase were prepared from *E. coli* MRE 600 according to the method of Burgess and Jendrisak [13] as subsequently modified [14]. Core polymerase was prepared by removal of the σ factor from the holoenzyme by chromatography over a Bio-Rex 70 column (Bio-Rad). Purified core enzyme was concentrated and stored at −80°C in storage buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.2 M NaCl and 50% glycerol). Protein purity was greater than 95% as judged by SDS–PAGE, and the level of residual σ^{70} was $<2\%$. Concentrations of core polymerase were determined spectrophotometrically using an extinction coefficient at 280 nm of 0.55 for a 1 mg/cm^3 solution and a molecular mass of 378 kDa [13].

2.2. Surface plasmon resonance

A BIAcoreX optical biosensor instrument was used with an NTA sensor chip (nitrilotriacetic acid bound to a solid support). SPR buffers and solutions were as follows: *eluent buffer*: 10 mM HEPES, 0.15 M NaCl, 0.05 mM EDTA, pH 7.4; *nickel solution*: 0.5 mM $NiCl_2$ in eluent buffer; *regeneration solution*: 10 mM HEPES, 0.15 M NaCl, 0.35 mM EDTA, pH 8.3.

The sensor chip contains two flow cells that can be run effectively in parallel to provide background corrections for non-specific binding of polymerase to the chip surface without bound σ factor. After extensive washing with regeneration buffer followed by eluent buffer, the two flow cells were loaded with nickel solution (20 μl) to saturate the NTA surface with Ni^{2+}. Following dialysis into eluent buffer, 20 μl of His-tagged σ^{70} (0.49 μM) was injected into flow channel 2, but not into the reference channel 1. 20 μl of core polymerase, which had been extensively dialysed against eluent buffer and brought to the appropriate concentration, was injected over both flow cells, and the sensorgram allowed to run for up to 30 min, following which the chip was regenerated. The procedure described by the manufacturer for regeneration did not, in our hands, result in a return to background signals, indicating that the surface had not been completely cleaned. However, the following four-step procedure was effective for complete regeneration. The chip was successively washed with 20 μl of the following solutions: (a) 0.3 M imidazole, (b) regeneration buffer, (c) 0.5% SDS, (d) 1 M NaOH, before equilibration for the next run. Two other practical matters are worthy of comment: first, that the His-tagged σ^{70} showed no binding to the NTA chip which had not previously been loaded with Ni^{2+}, showing that binding was exclusively via the histidine tag; second, that the core RNA polymerase did show some non-specific binding to the Ni–NTA chip in the absence of σ factor, which could be corrected by subtracting the sensor signal of the reference channel 1 from the experimental binding data observed in channel 2.

3. Results and discussion

It has been noted previously that some His-tagged proteins, where the tag contains only a single group of six histidine residues, can show slow leaching from the Ni–NTA surface in SPR work, particularly at high concentrations [15]. This was observed with the σ factor in the present work, and extensive preliminary studies were carried out to establish the appropriate loading density for σ factor to the chip surface to minimise this problem. Levels of σ corresponding to 50–400 RU (resonance units) gave stable baselines, and enough bound σ to observe polymerase binding. (1 RU corresponds approximately to a protein loading of 1 pg/mm^2 on the sensor chip.) For every binding experiment, a correction for non-specific binding was applied by subtraction of the reference channel data as described above. The correction was obtained at the same time and with the same polymerase solution as was used to determine binding, and control experiments established that the correction was very precise. The magnitude of this correction was about 10–20% of the specific binding signal, depending on the concentration of core polymerase used. Fig. 1 illustrates the corrected overlay plots (channel 2 minus channel 1) for a series of binding experiments when the chip was loaded with a density of 500 RU; the signal produced by core binding increases with polymerase concentra-

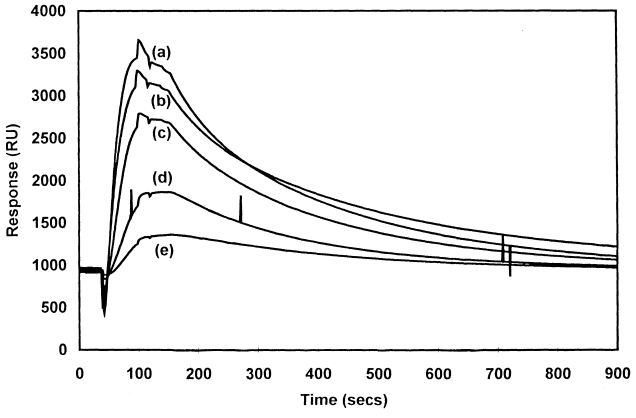

Fig. 1. Overlay plots showing the binding of core RNA polymerase to His-tagged σ^{70} immobilised on the Ni^{2+}–NTA surface. The flow rate was 20 μl/min. The signal for binding of the ligand (σ^{70}) was 500 RU. Core polymerase was passed over the chip for about 100 s, before initiating the dissociation phase by washing with buffer. The polymerase concentrations were 1.05 μM (a and b), 0.525 μM (c), 0.21 μM (d) and 0.105 μM (e). Kinetic analysis was carried out on the dissociation phases of the traces, yielding dissociation rate constants of: (a) $4.6(\pm 0.5)\times 10^{-3}$ s^{-1}, (b) $4.2(\pm 0.5)\times 10^{-3}$ s^{-1}, (c) $4.2(\pm 0.7)\times 10^{-3}$ s^{-1}, (d) $3.9(\pm 1.0)\times 10^{-3}$ s^{-1}, (e) $3.6(\pm 1.5)\times 10^{-3}$ s^{-1}.

tions, and following the dissociation phases when the chip is washed with buffer, the signal returns to the initial levels.

The response signal just prior to dissociation of the core from σ^{70}, when the flow of core stops, was plotted against core concentration in Fig. 2. The data are plotted as fractional saturation parameter (θ) which is given by $\Delta RU/\Delta RU_{max}$ where ΔRU is the change in response observed, and ΔRU_{max} is the value corresponding to saturation with core. The fractional saturation parameter is easier to use for global analysis of data from several experiments in which the initial loading and hence the values of ΔRU_{max} are different. The data follow a typical hyperbolic binding curve, which was fitted by non-linear regression to the following function that describes a single-site binding model [17]

$$\theta = [\text{core}]/(K_D + [\text{core}])$$

where θ is the fractional saturation parameter defined above, and K_D is the dissociation constant for the core–σ interaction. Since the magnitude of the SPR signal is proportional to the mass of the entity bound on the chip surface, the saturating levels of ΔRU_{max} can be used in combination with the change in signal on loading the chip with σ^{70} (ΔRU_s) and the known molecular weights of the core (M_c) and σ (M_s) proteins to evaluate the stoichiometry (n) of binding according to the following equation:

$$n = (\Delta RU_{max}/\Delta RU_s)/(M_s/M_c)$$

Values of the stoichiometry determined in this way were 0.90 ± 0.15, which is consistent with 1:1 binding of σ to core and suggests that the proteins have a high level of functional activity in specific σ–core recognition.

The globally fitted value of K_D determined from the data, on the basis of a 1:1 stoichiometry, was 1.9×10^{-7} M with a 67% confidence range of 1.1×10^{7}–2.7×10^{-7} M, calculated from the sum of the squares procedure of Hoare [16].

The binding constant for the interaction of σ^{70} with core polymerase determined in the present work is somewhat lower than the value suggested from previous fluorescence studies with pyrene-labelled σ^{70} which seemed to be compatible with binding tighter than about 10^{-9} M. In the earlier work, the interaction was studied in a medium containing 5% glycerol, and there are grounds for believing that glycerol can strengthen protein–protein interactions. Unfortunately, we have not found it possible to introduce glycerol in the present SPR studies for practical reasons. It is also possible that the large pyrene group used in the earlier work may affect binding quantitatively. The stoichiometric concentrations of σ^{70} and core polymerase in *E. coli* are about 1.5 μM and 3 μM respectively, which is much greater than the observed binding constant, and would therefore allow the formation of stable holoenzyme. In terms of RNA polymerase function, the holoenzyme needs to form stably for promoter recognition and initiation, but too tight an interaction between core and σ might serve to slow down σ release and promoter escape.

In principle, the data from the sensorgrams can be used to evaluate the kinetic parameters of binding, although it is well recognised that such analysis is not straightforward when the interaction is a multi-step process. This is the case in binding of σ to core, where initial rapid bimolecular association is followed by slower conformational changes [9,10]; the association process will be kinetically biphasic, and may well be affected by mass transport [11]. Evaluation of the dissociation phase is usually simpler, and essentially represents exponential dissociation of the complex limited by the slowest step. We have not in the present work carried out a complete kinetic analysis of the multi-step association and dissociation process. However, the dissociation phases of the five sensorgrams in Fig. 1 all fitted well ($\chi^2 = 0.02$, $R = 0.95$) to a single exponential decay function, and on the basis that this represents a single dissociation process, the mean dissociation rate constant was evaluated to be 4.0 (± 1.0)$\times 10^{-3}$ s^{-1}.

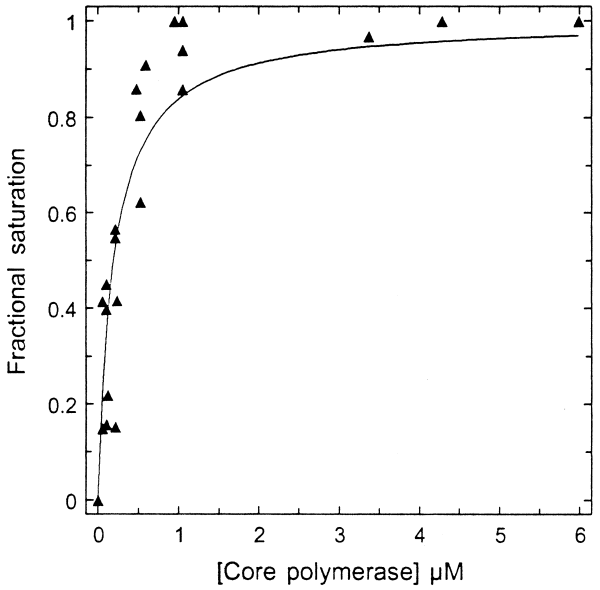

Fig. 2. Binding of core polymerase to His-tagged σ^{70} immobilised on a Ni^{2+}–NTA sensor chip. Fractional saturation was calculated as described in the text. The data were fitted by non-linear regression to a hyperbolic binding function describing a single-site binding model (see text).

Although the kinetics of σ–core interactions have been studied for σ^{70} [10] and σ^{N} [9] and it has been demonstrated that in both cases σ induces conformational changes in the resulting holoenzymes, little direct information exists on the σ release step, which has been assumed to be relatively slow. The kinetic results presented here support this assumption, and suggest that, in the absence of DNA or transcription, the half-life of the σ^{70}–core complex is about 200 s. Our results are in broad agreement with those of Fenton et al. [12], who observed half-lives in the range 4–11 min for wild-type and a range of mutant σ^{70} factors.

The results presented here show that SPR can be used successfully to investigate quantitatively protein interactions in transcription complex assembly, utilising histidine tagging to immobilise protein components on the SPR chip. Conventional histidine tagging uses six His residues, which is sufficient to hold the protein in the Ni–NTA chip surface, provided the (immobilised) ligand loading is not too high. However, there are advantages in terms of more stable ligand binding and consequently sensorgram baselines in having longer or multiple tagging regions ([14], and Skinner, G.M. and Hoggett, J.G., unpublished observations).

We are presently extending these SPR studies to other σ factors to assess their affinities for the core enzyme, and to

investigate the competition between the different factors which is important in coordinating gene regulation in bacteria.

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