

Quantitative analysis of the ternary complex of RNA polymerase, cyclic AMP receptor protein and DNA by fluorescence anisotropy measurements[★]

Piotr Bonarek[✉], Sylwia Kędracka-Krok, Barbara Kępyś and Zygmunt Wasylewski

Department of Physical Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

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The *in vitro* formation of transcription complexes with *Escherichia coli* RNA polymerase was monitored using fluorescence anisotropy measurements of labeled fragments of DNA. The multicomponent system consisted of holo or core RNA polymerase (RNAP) and *lac* or *gal* promoter fragments of DNA (in different configurations), in the presence or absence of CRP activator protein (wt or mutants) with its ligand, cAMP. Values of the apparent binding constants characterizing the system were obtained, as a result of all processes taking place in the system. The interaction of the promoters with core RNAP in the absence of CRP protein was characterized by apparent binding constants of 0.67 and $1.9 \times 10^6 \text{ M}^{-1}$ for *lac166* and *gal178*, respectively, and could be regarded as nonspecific. The presence of wt CRP enhanced the strength of the interaction of core RNAP with the promoter, and even in the case of *gal* promoter it made this interaction specific (apparent binding constant $2.93 \times 10^7 \text{ M}^{-1}$). Holo RNAP bound the promoters significantly more strongly than core RNAP did (apparent binding constants 1.46 and $40.14 \times 10^6 \text{ M}^{-1}$ for *lac166* and *gal178*, respectively), and the presence of CRP also enhanced the strength of these interactions. The mutation in activator region 1 of CRP did not cause any significant disturbances in the holo RNAP–*lac* promoter interaction, but mutation in activator region 2 of the activator protein substantially weakened the RNAP–*gal* promoter interaction.

Keywords: RNA polymerase, cAMP receptor protein, fluorescence anisotropy

INTRODUCTION

Transcription activation by cAMP receptor protein (CRP) from *Escherichia coli* is a simple example of transcription activation (Busby & Ebright, 1999). CRP-dependent promoters require only three components: a specific DNA sequence, RNA polymerase enzyme (RNAP), and CRP. RNAP con-

sists of five subunits: α , which assembles into a dimer, β , β' , σ and ω , and it exists in two forms: core- and holo-enzyme. The about 400 kDa core possesses an enzymatic activity necessary for transcription, but it can not recognise specific promoter DNA sequence. The full activity is a result of the interaction of the core with one type of σ subunits. The 70-kDa σ^{70} subunit is the most abundant one and it is the

[✉]Corresponding author: Piotr Bonarek, Department of Physical Biochemistry, Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland; tel.: (48 12) 664 6151; fax: (48 12) 664 6902; e-mail: piotr.bonarek@uj.edu.pl

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Abbreviations: bp, base pair; cAMP, 3',5'-cyclic adenosine monophosphate; CRP, cAMP receptor protein; AR, activating region; α CTD, C-terminal domain of α subunit of RNA polymerase; α NTD, N-terminal domain of α subunit of RNA polymerase; DTT, dithiothreitol; *gal178*, labeled fragment of promoter *gal* from *Escherichia coli*; IPTG, isopropyl β -D-1-thiogalactopyranoside; *lac166*, labeled fragment of promoter *lac* from *Escherichia coli*; Ni^{2+} -NTA, Ni^{2+} -nitrilotriacetate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; RNAP, RNA polymerase; SDS, sodium dodecyl sulfate; wt, wild type.

focus of the present studies. Holo RNAP can bind to specific and nonspecific DNA sequences (deHaseth *et al.*, 1978). The nonspecific interaction allows for a faster search of specific sites by one-dimensional diffusion of RNAP molecule along the DNA template (Kabata *et al.*, 1993; Sakata-Sogawa *et al.*, 2004). Initially, the specific promoter sequence and RNAP form a closed complex which is followed by a series of isomerisation steps. Finally, the RNAP-promoter open complex is created which contains separated strands of double helical DNA and enters the elongation phase (Craig *et al.*, 1998; deHaseth *et al.*, 1998; Saecker *et al.*, 2002; Tsujikawa *et al.*, 2002).

CRP (also called the catabolite gene activator protein – CAP) is a homodimer, and both its subunits consist of two domains (Aiba *et al.*, 1982). The large N-terminal domain binds an allosteric factor: cAMP. The minor C-terminal domain recognises specific DNA sites by a helix-turn-helix motif (McKay & Steitz, 1981; Weber & Steitz, 1987; Passner *et al.*, 2000). CRP controls over one hundred promoters (Kolb *et al.*, 1993) that are grouped into three classes: I, II and III (Ushida & Aiba, 1990; Ebright, 1993; Busby & Ebright, 1999).

The presence of CRP enhances the promoter–RNAP interaction by creating additional contacts between the activator and other complex components (Malan *et al.*, 1984; Ren *et al.*, 1988; Straney *et al.*, 1989). The number of contacts is different for each promoter class. Only one molecule of CRP is required for initiation of transcription with class I and II CRP-dependent promoters and its DNA site is located upstream of the transcription start point. At class I promoters CRP binds upstream of the site for RNAP and can be centered near positions –93, –83 or –62 (Zhou *et al.*, 1994; Naryshkin *et al.*, 2000). The CRP site overlaps the site for RNAP on class II promoters. The best-characterized representatives of the class I and II promoters are the *lac* and the *gal* ones, for which the CRP sites are centered at positions –61.5 and –41.5, respectively. Class III CRP-dependent promoters require more than one activator molecule.

Transcription activation at the *lac* promoter requires formation of a contact between one α C-terminal domain and CRP. The CRP determinant consists of residues 156–164 (AR1). T158 is the most important side-chain for function of AR1 (Niu *et al.*, 1994). Residues 285–289 and 315–318 form the α subunit “287 determinant”. The second “265 determinant” (R265, N268, N294, G296, K298, S299, E302), essential for DNA binding, is responsible for recognition of a specific sequence on the promoter (Gaal *et al.*, 1996; Murakami *et al.*, 1996). The crystallographic structure of the α CTD–CRP–DNA complex confirms the described mechanism (Benoff *et al.*, 2002).

In addition to those mentioned above, transcription activation of the *gal* promoter requires two additional interactions of CRP with RNAP (Busby & Ebright, 1999). AR1 of the upstream subunit of CRP interacts with the 287 determinant of α CTD (Bell *et al.*, 1990; Williams *et al.*, 1991; Savery *et al.*, 1998). AR2, a second CRP determinant consisting of residues H19, H21 and K101 interacts with α NTD determinant, which consists of residues 162–165 (Niu *et al.*, 1996). The active AR2 sequence is located on the distal CRP monomer. It seems that this protein–protein interaction is an electrostatic one between the negative charge on the “165 determinant” and the positive charge on AR2.

AR3, a third CRP determinant consisting of D53, E54, E55 and E58 interacts with residues 593–603 of σ^{70} subunit. This interaction has also an electrostatic nature, for which the contact between E58 of AR3 and two arginines of σ^{70} plays a key role (Rhodius & Busby, 2000a; 2000b).

As a consequence of the above-mentioned studies and with the use of crystallographic data, molecular models of transcription activation by CRP on *lac* and *gal* promoters were generated (Lawson *et al.*, 2004).

However, numerous molecular details concerning these processes are still unknown. Only the knowledge of binding constants enables one to predict the behavior of the molecular system studied at fixed concentrations of reagents. In this report we describe the use of fluorescence anisotropy method to quantitatively analyze the complicated molecular system comprising: RNAP, CRP and DNA. Our research model has three important advantages: i) all measurements are conducted in solution, ii) its sensitivity allows the use of low concentrations of macromolecules, and iii) no protein structure has been modified. Therefore, conditions closely resembling the *in vivo* state could be simulated. The applied model not only enabled us to confirm earlier results, but also provided new information concerning the role of CRP in regulation of the gene transcription.

MATERIALS AND METHODS

Buffers. All buffers were prepared from deionized water from a MiliQ system and highest grade chemicals. Buffer A, 50 mM NaH_2PO_4 , pH=7.0, containing 5 mM imidazole and 500 mM NaCl. Buffer B, 40 mM Tris/HCl, pH=7.9, containing 300 mM KCl and 10 mM EDTA. Buffer C, 50 mM Tris/HCl, pH=7.9, containing 100 mM KCl, 1 mM EDTA and 5% (v/v) glycerol. Buffer D, same as buffer B, but containing 20% glycerol. Buffer E, 50 mM Tris/HCl, pH=7.9, containing 100 mM KCl and 10% (v/v) gly-

erol. Buffer F, 10 mM Tris/HCl, pH=8.0, containing 0.1 mM EDTA

Protein expression and purification

Expression of RNAP subunits. The β , β' , σ^{70} and the N-terminal His-tagged α polymerase subunits were purified from *E. coli* BL21(DE3) carrying plasmids: pHTT7f1-NH α (*rpoA*) (Tang *et al.*, 1995) for α subunit, pMKSe2 (*rpoB*) (Severinov *et al.*, 1993) or pET21a+(*rpoB*) for β subunit, pT7 β' (*rpoC*) (Zalenskaya *et al.*, 1990) or pET28a+(*rpoC*) for β' subunit and pET-21 σ (*rpoD*) (Hernandez *et al.*, 1996) for σ^{70} subunit. Plasmid pET21a+(*rpoB*) was constructed by transferring *rpoB* gene to commercial plasmid pET21a (Novagen) using restriction enzymes: *NdeI/HindIII*. Plasmid pET28a+(*rpoC*) was constructed by transferring *rpoC* gene to commercial plasmid pET28a (Novagen) using restriction enzymes: *NcoI/XhoI*.

Bacterial strains were grown in LB broth with ampicillin at 37°C until OD₆₀₀ reached 0.8, then protein expression was induced with 1 mM IPTG. The cultures were shaken for additional 3 h and harvested by centrifugation. The cell pellets were frozen at -80°C.

Purification of α subunit. Frozen cell pellet was resuspended in 60 ml of buffer A plus 1 mM PMSF, 1 mM DTT, 0.2 mg/ml lysozyme, mixed to homogeneity and after incubation for 20 min, sonicated eight times with 30 s pulses at the maximum power setting using the Techpan UD-11 sonicator. The mixture was centrifuged at 30000 \times g, 30 min at 4°C, and the supernatant was loaded at 1 ml/min on a 10-ml Ni²⁺-NTA agarose column (Novagen) preequilibrated with buffer A. The column was washed with 50 ml of buffer A, the bound proteins were eluted with 20 ml of buffer A plus 300 mM imidazole. Two-milliliter fractions were collected and aliquots of each fraction were analyzed by SDS/12% PAGE. The fractions containing α subunit (about 90% purity) were pooled, dialysed overnight against 2 l of buffer D and stored at -30°C.

Purification of β , β' and σ^{70} subunits. Purification of inclusion bodies formed by all three subunits was conducted according to the method of Tang *et al.* (1996) with one modification. A 0.2% (v/v) Triton X-100 was used in place of n-octyl- β -D-glucoside. Washed inclusion bodies were resuspended in buffer B plus 10% (v/v) glycerol and stored at -80°C.

Reconstitution and purification of RNAP. The enzyme was reconstituted from subunits as described previously (Tang *et al.*, 1996). The core or holo RNAP was loaded at 2 ml/min on a 10-ml Ni²⁺-NTA column equilibrated with buffer E using a peristaltic pump. The column was then washed with 40 ml of the same buffer and the bound proteins were eluted with 25 ml of buffer E with 100 mM imidazol.

The fractions containing the required proteins were pooled, diluted four times with buffer C and loaded at 3 ml/min on a MonoQ 10/10 HR column preequilibrated with buffer C using Äkta Explorer FPLC system. The column was washed with 50 ml of buffer C with 0.2 M KCl. The bound proteins were eluted with a gradient of KCl in buffer C (0.2–0.5 M KCl in 100 ml). Aliquots of 10 μ l of the fractions were analysed by 7.5–12% SDS/PAGE. The fractions containing pure RNAP core (>98%) were pooled and dialysed overnight against 0.5 l of buffer C or storage buffer (50 mM Tris/HCl, pH=7.9, containing 100 mM KCl, 1 mM EDTA and 50% (v/v) glycerol). The fractions in storage buffer were stored at -80°C.

Purification of CRP. CRP wt and three point mutants: H19A, H21A, T158A were isolated from *E. coli* M182 Δ *crp* (Busby *et al.*, 1983) transformed with pHA7 plasmids (Aiba *et al.*, 1982) carrying respective mutant *crp* genes. The mutations were inserted using the overlap extension method with *Pwo* polymerase. Cells were grown in LB medium with ampicillin at 37°C. All proteins were purified as described previously (Malecki *et al.*, 2000). The obtained protein solutions indicated >97% purity as judged by SDS/PAGE with Coomassie Brilliant Blue staining.

Amplification and purification of DNA fragments from PCR mixture. A 166-bp DNA fragment of *lac* promoter (*lac166*) labeled at 5'-end with fluorescein was synthesized by amplification of a fragment of pUC19 plasmid in a PCR reaction using the following primers:

for -5'-GTA ATC ATG GTC ATA GCT GTT TCC-3',
rev -5'-fluorescein-CAG GTT TCC GGA CTG GAA AGC-3',

and a 178-bp DNA fragment labeled at 5'-end with fluorescein containing *gal* promoters P₁ and P₂ (*gal178*) was synthesized by amplification of a fragment of pSA509 plasmid (Choy & Adhya, 1992) in a PCR reaction using the following primers:

for -5'-fluorescein-CGG CTA AAT TCT TGT GTA AAC-3',
rev -5'-ATT TGC TGC AGT AAT TGC ACA-3'.

Thus, the fluorescent dye was placed about 60 nucleotides upstream of the CRP-binding site.

The PCR mixture was 4-fold diluted with buffer F and loaded on a MonoQ 10/10 HR column pre-equilibrated with the same buffer F. After loading it was washed with 20 ml of buffer F and a gradient of NaCl (in buffer F) from 0 to 1.0 M was used to elute the DNA fragment. Separation of the obtained fractions was monitored by absorbance measurement at 260 nm and ethidium bromide-stained agarose gel electrophoresis of DNA samples.

Fluorescence anisotropy measurements. The experiments were performed with a Fluorolog-3 spectrofluorometer (JY Horiba) using photon counting technique and an L-format setup. The configura-

tion of monochromators was 1:2 (i.e., single-grating at excitation, double-grating at emission position). Samples containing fluorescein-labeled oligodeoxyribonucleotides were excited at 490 nm. The intensity of fluorescence emission at 520 nm at vertical-vertical (I_{VV}) and vertical-horizontal (I_{VH}) directions was determined. The fluorescence anisotropy (r) was calculated according to equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

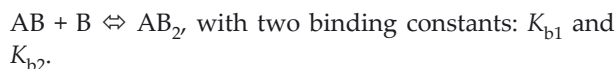
The G-factor (defined as the ratio of fluorescence intensity measured in horizontal-vertical (I_{HV}) to horizontal-horizontal (I_{HH}) polarizer positions) was determined in preliminary measurements and remained fixed as a constant value during proper experiments. The chosen excitation and emission bandpasses depended on sample concentration and gave the intensity of fluorescence in all polarizer positions within the range $0.5\text{--}1.5 \times 10^6$ cps. Temperature of $25.0 \pm 0.1^\circ\text{C}$ was maintained by a Julabo F25 water-circulated thermostat chamber. Samples were incubated at 25°C for 30 min before conducting experiments. All measurements were carried out in a 10 mm-pathlength quartz cuvette in titration buffer C. Before measurements all components were simultaneously dialysed against the same portion of titration buffer. Afterwards, the samples were centrifuged ($25000 \times g$, 10 min, 20°C) and the concentrations were determined using Bradford reagent for RNAP and extinction coefficients of $\epsilon_{278} = 40800 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{259} = 14650 \text{ M}^{-1} \text{ cm}^{-1}$ for CRP and cAMP, respectively. The DNA concentration was calculated assuming $A_{260} = 1.0$ corresponds to dsDNA concentration of $50 \mu\text{g/ml}$; the molecular mass of the examined promoter fragments was 650 Da/bp on average. The titrations were performed by adding to *lac166* or *gal178* solution ($1800 \mu\text{l}$ of $20\text{--}40 \text{ nM}$) small aliquots ($2\text{--}20 \mu\text{l}$) of the stock solution of holo or core RNA polymerase ($5\text{--}10 \mu\text{M}$) containing DNA in appropriate concentration (the same as in the solution being titrated). All measurements were carried out in the presence of $200 \mu\text{M}$ cAMP which is more than sufficient to saturate the available anti-periplanar binding sites of CRP. When the experiment was carried out with CRP protein, the initial sample and titrating solution also included it. After each addition the solution was stirred and equilibrated to reach the desired temperature. Measured anisotropy represented the mean value of at least two repetitions, which in turn involved 3–5 accumulation points. A single experiment included about 30 additions which corresponded to about 20-fold molar excess of RNAP over DNA.

Calculations. Two binding models of the studied interactions were used. The first model,

named B, described the mechanism of the reaction according to equation:



The second one, named BB, described the mechanism of the reaction according to equations:



More sophisticated models did not give a better fit, but generated large discrepancies and errors of the fitted parameters. Besides, the sequence model of binding was more preferable. Estimation of the parameters for systems of equations was performed with the program DynaFit (Kuzmic, 1996) using data obtained experimentally.

RESULTS

DNA–holo RNAP interaction and CRP influence on transcription complex formation

We observed an increase of fluorescence anisotropy of labeled DNA with increasing RNAP concentration, in the absence or presence of CRP. Additionally, the increase of the anisotropy was sensitive to the composition and concentration of the reagents.

During titration experiments up to 5-fold increase of fluorescence anisotropy was observed. The influence of protein binding on the fluorescence emission spectrum of DNA was also checked. The addition of CRP (or its mutants) did not change the fluorescence intensity, while the presence of RNAP holoenzyme caused a 20% and 25% decrease in fluorescence intensity for *lac166* and *gal178*, respectively (not shown). The analysis of total fluorescence changes (according to the sum: $I_{VV} + 2GI_{VH}$) also confirmed this result.

Table 1. Binding parameters obtained for *lac166*–holo RNAP interaction according to model B at various concentrations of CRP variants

Protein	[CRP] (nM)	K_{app} (10^6 M^{-1})	r_{lac166}	$r_{lac166\text{holo}}$
CRPwt	0	1.46 ± 0.15	0.051 ± 0.001	0.286 ± 0.013
	400	3.69 ± 0.32	0.051 ± 0.002	0.225 ± 0.005
	860	4.78 ± 0.36	0.054 ± 0.002	0.216 ± 0.003
	2000	3.86 ± 0.30	0.060 ± 0.002	0.225 ± 0.004
	0	3.40 ± 0.42	0.041 ± 0.003	0.181 ± 0.005
CRPT158A	400	2.74 ± 0.30	0.044 ± 0.002	0.187 ± 0.005
	800	4.77 ± 0.49	0.042 ± 0.002	0.171 ± 0.003
	2000	6.22 ± 0.50	0.052 ± 0.002	0.166 ± 0.002

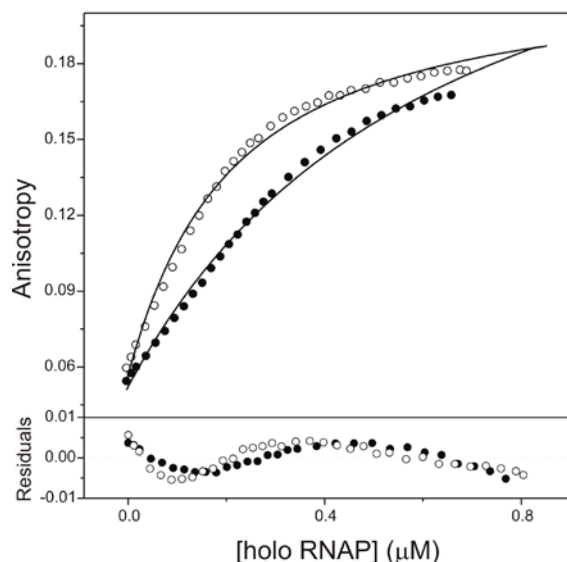


Figure 1. Fluorescence anisotropy analysis of holo RNAP-*lac166* interactions in the absence of CRP (●) and at 860 nM CRP wt (○).

Solid lines are representative fitting curves according to model B. The goodness of fit is shown by distribution of residuals (lower panel).

The study of transcription complex formation between holo RNAP and *lac166* was performed in the absence of activator protein or at increasing concentrations of CRP wt or mutant T158A (400 nM, 800 nM and 2000 nM). In all cases an increase of fluorescence anisotropy during titration was observed, ranging from 0.04–0.06 to 0.14–0.18 (Fig. 1). The fluorescence anisotropy increased more rapidly in the presence of CRP than in its absence.

The measured changes for *lac166* in all cases could be satisfactorily fitted by the model B:

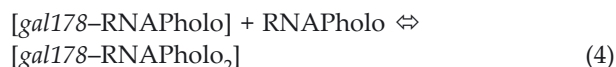


The solid line in Fig. 1 is an example of the fitting procedure according to model B. The obtained values of the binding constant K_{app} and the calculated theoretical anisotropy for free *lac166* (r_{lac166}) and *lac166* bound to one molecule of holoenzyme ($r_{lac166holo}$) are depicted in Table 1.

A series of titration experiments were also performed for *gal178* fragment without CRP or with one of the following: CRPwt, H19A or H21A. The observed anisotropy values strongly depended on the concentration of holo RNAP and also on of CRP. The range of anisotropy changes at a given CRP concentration reached a few hundred percent (see Fig. 2). The initial value of fluorescence anisotropy for *gal178* was of 0.05, similar to the value obtained for *lac166*. However, the final anisotropy was highly variable. The biggest changes were observed in the absence of CRP. Even a small amount

of CRP significantly decreased the amplitude of the change. A correlation between the initially observed anisotropy value and CRP concentration was also observed. It was probably a result of nonspecific protein–DNA interactions. This could also explain the smallest changes of anisotropy at the highest CRP concentration (2 μ M), about 180% for CRPwt and 220% for the mutants. Increasing concentration of CRP changed the shape of the titration curves especially at low RNAP concentrations. The bending effect was significant in the case of mutants and was smaller for CRPwt at a 5-fold molar RNAP excess over DNA. More plain anisotropy changes of the shape were observed at higher, over 1 μ M, CRP concentration.

The titration curves described above were analyzed with the DynaFit program according to model BB, which corresponded to the following reactions:



The averaged values of association constants of $4.014 \pm 0.935 \times 10^7 \text{ M}^{-1}$ and $7.7 \pm 2.3 \times 10^5 \text{ M}^{-1}$ obtained for experiments without CRP and with CRP could be related to specific and nonspecific holoenzyme-*gal178* interaction, respectively. The presence of CRP made the analysis much more difficult. However, the values of K_{1ass} and K_{2ass} increased with increasing CRP concentration, calculated values of anisotropy parameters: r_{gal178} , $r_{gal178holo}$, $r_{gal178holo2}$ were often unlikely or incorrect (close to zero or >0.4). Also the binding constants reached very high values under the same conditions. The distribution of the residuals in the range of low RNAP concentrations indicated the existence of an interaction characterized by a high association constant, which could not be determined correctly by the BB model. We decided to modify the BB model by introduction of a fixed (equal to 10^9 M^{-1}) first binding constant in order to limit the number of fitted parameters and to simplify the calculations. This value of the constant was the lowest one, which made it possible to obtain coherent results. Specified in this way, the BB model better described the titration curves obtained for the interaction of holo RNAP with *gal178* in the presence of CRP (Fig. 2 and Table 2).

DNA-core RNAP interaction in the presence or absence of CRP

Similar titration experiments were performed for the interaction of core RNAP with DNA. These measurements were done only for CRPwt. The

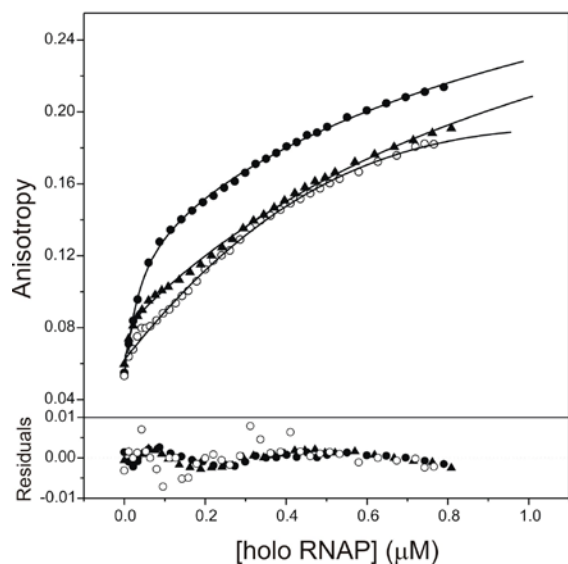
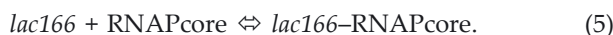


Figure 2. Isotherms of holo RNAP–*gal178* interactions in the absence of CRP (●), at 100 nM (○) and 800 nM (▲) CRP wt.

Solid lines are representative fitting curves according to model BB. The goodness of fit is shown by the distribution of residuals (lower panel).

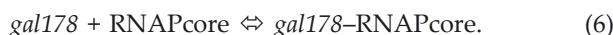
quantum yield of fluorescence changed during titration in the range of 20%.

The interaction of core RNAP with *lac166* was accompanied by anisotropy changes smaller (about 300%) than those caused by the holoenzyme. The presence of CRP caused an increase of fluorescence anisotropy changes at each point of the titration curve as well as an increase of the final anisotropy (compare in Fig. 3). The simplest model B described the experimental data very well regardless of the presence of CRP.



The obtained parameters are shown in Table 3. The average value of the association constant in the presence of CRP was $1.4 \times 10^7 \text{ M}^{-1}$, about twice higher than without the activator protein, however, the calculated anisotropy values of studied system did not change.

The experimental data obtained for the interaction of core RNA with *gal178* in the absence of CRP could also be adequately fitted by model B (see black points on Fig. 4):



The calculated binding parameters are presented in Table 4. However, the addition of the ac-

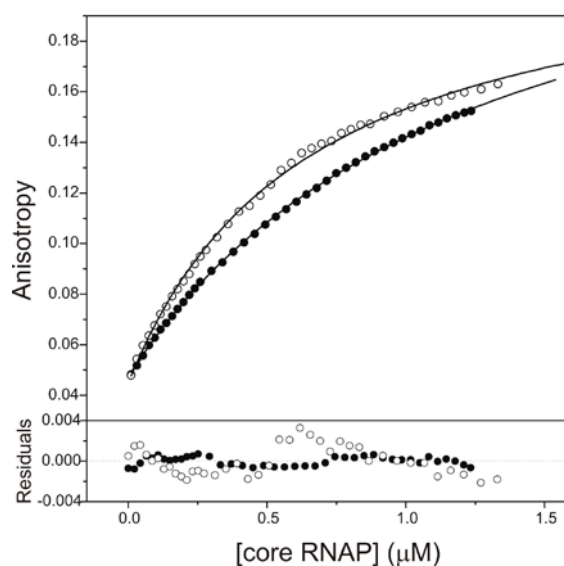
Table 2. The binding parameters obtained for *gal178*–holo-RNAP interaction according to BB model at various concentrations of CRP variants[†]

Protein	[CRP] (nM)	K_{1app} (10^6 M^{-1})	K_{2app} (10^6 M^{-1})	r_{gal178}	$r_{gal178holo}$	$r_{gal178holo2}$
	0	40.14 ± 9.35	0.77 ± 0.23	0.052 ± 0.002	0.126 ± 0.008	0.348 ± 0.033
CRPwt	10	1000	1.29 ± 0.08	0.053 ± 0.001	0.075 ± 0.001	0.301 ± 0.008
	30	1000	1.05 ± 0.09	0.052 ± 0.002	0.067 ± 0.001	0.315 ± 0.012
	100	1000	1.26 ± 0.11	0.055 ± 0.002	0.069 ± 0.001	0.309 ± 0.011
	400	1000	1.88 ± 0.13	0.057 ± 0.002	0.077 ± 0.001	0.293 ± 0.007
	800	1000	1.40 ± 0.12	0.059 ± 0.002	0.074 ± 0.001	0.305 ± 0.011
	1200	1000	0.83 ± 0.08	0.060 ± 0.001	0.085 ± 0.001	0.361 ± 0.016
	1600	1000	1.64 ± 0.10	0.062 ± 0.001	0.082 ± 0.001	0.281 ± 0.006
	2000	1000	1.99 ± 0.09	0.101 ± 0.001	0.121 ± 0.001	0.272 ± 0.003
CRPH19A	10	60 ± 12	0.42 ± 0.17	0.048 ± 0.001	0.119 ± 0.004	0.406 ± 0.083
	30	122 ± 41	0.35 ± 0.17	0.052 ± 0.002	0.105 ± 0.003	0.489 ± 0.140
	100	166 ± 77	0.19 ± 0.23	0.051 ± 0.002	0.103 ± 0.004	0.714 ± 0.643
	800	151 ± 37	0.80 ± 0.13	0.059 ± 0.001	0.110 ± 0.002	0.337 ± 0.021
	1200	598 ± 156	1.44 ± 0.09	0.075 ± 0.001	0.120 ± 0.001	0.287 ± 0.005
	1600	242 ± 68	1.16 ± 0.13	0.067 ± 0.001	0.115 ± 0.002	0.314 ± 0.011
	2000	1000	1.75 ± 0.10	0.088 ± 0.001	0.114 ± 0.001	0.263 ± 0.004
CRPH21A	10	41 ± 17	0.76 ± 0.30	0.052 ± 0.002	0.107 ± 0.008	0.300 ± 0.041
	100	1000	0.53 ± 0.08	0.053 ± 0.002	0.081 ± 0.001	0.402 ± 0.036
	400	697 ± 745	0.46 ± 0.11	0.057 ± 0.002	0.083 ± 0.002	0.467 ± 0.070
	800	116 ± 60	0.16 ± 0.13	0.059 ± 0.002	0.093 ± 0.003	0.932 ± 0.586
	1200	437 ± 381	0.58 ± 0.16	0.060 ± 0.002	0.092 ± 0.003	0.384 ± 0.055
	1600	1000	1.17 ± 0.12	0.062 ± 0.002	0.092 ± 0.001	0.314 ± 0.013
	2000	1000	1.42 ± 0.14	0.078 ± 0.002	0.104 ± 0.001	0.267 ± 0.008

[†]Fixed value of K_{app1} equal to $1 \times 10^9 \text{ M}^{-1}$ was used in some analyses (for explanation, see Results).

Table 3. Binding parameters obtained for *lac166*-core RNAP interaction according to model B

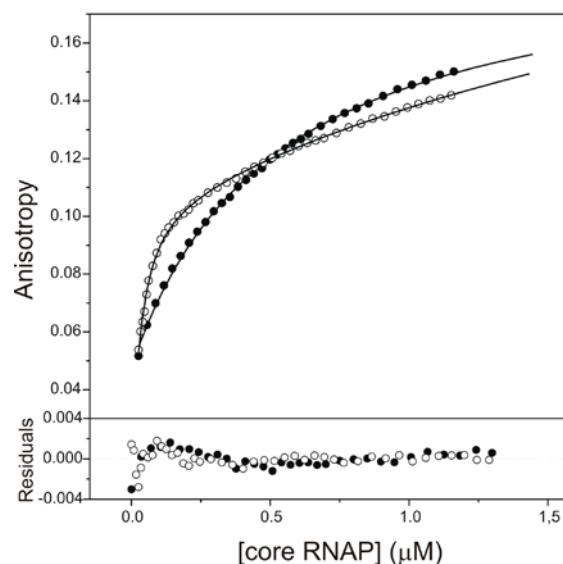
[CRPwt] (nM)	K_{app} ($10^6 M^{-1}$)	r_{lac166}	$r_{lac166core}$
0	0.75 ± 0.03	0.048 ± 0.000	0.274 ± 0.005
	0.82 ± 0.01	0.049 ± 0.000	0.257 ± 0.002
	0.44 ± 0.06	0.047 ± 0.000	0.145 ± 0.009
100	1.56 ± 0.06	0.047 ± 0.001	0.222 ± 0.003
	1.25 ± 0.04	0.049 ± 0.001	0.223 ± 0.003

**Figure 3. Isotherms of core RNAP-*lac166* interactions in the absence of CRP (●) and at 100 nM (○) CRP wt.**

Solid lines are representative fitting curves according to model B. The goodness of fit is shown by the distribution of residuals (lower panel).

tivator protein changed the course of the titration curves and made model B improper. The BB model turned out good enough to describe the obtained data (see open points on Fig. 4).

The average value of the association constant for the core RNAP-*gal178* interaction measured without CRP was two times higher than for *lac166*. In the presence of 100 nM CRPwt the first binding constant for the core RNAP-*gal178* interaction was equal to $2.93 \times 10^7 M^{-1}$ and was 100-fold higher than the second one (Table 4).

**Figure 4. Isotherms of core RNAP-*gal178* interactions in the absence of CRP (●) and at 100 nM (○) CRP wt.**

The solid line corresponds to the best fit according model B in the absence of CRP, and according to model BB in the presence of CRP. The goodness of fit is shown by distribution of residuals (lower panel).

DISCUSSION

The initial value of the fluorescence anisotropy was in the range from 0.04 to 0.06, and increased in the presence of CRP. The specific interaction between CRP and DNA is quite strong, the association constants have been reported in the range from 10^7 to $10^{10} M^{-1}$ (Ebright *et al.*, 1989; Takahashi *et al.*, 1989; Heyduk & Lee, 1990; Pyles & Lee, 1998; Leu *et al.*, 1999), which indicates that even a low CRP concentration causes saturation of the specific sequence. On the other hand, the association constant of a nonspecific interaction was reported at about $10^4 M^{-1}$ (Giraud-Panis *et al.*, 1994). Hence, the increase of anisotropy in the presence of high, micromolar CRP concentration was a result of nonspecific interactions.

The final values of the observed anisotropy were in the range from 0.14 to 0.18. These values result from an immobilization of DNA ends, an effect

Table 4. The binding parameters obtained for *gal178*-core RNAP interaction in the absence of CRPwt according to model B and in the presence of CRP wt according to model BB[†]

[CRPwt] (nM)	K_{1app} ($10^6 M^{-1}$)	K_{2app} ($10^6 M^{-1}$)	r_{gal178}	$r_{gal178core}$	$r_{gal178core2}$
0	1.66 ± 0.05	-	0.055 ± 0.001	0.194 ± 0.002	-
	2.13 ± 0.08	-	0.056 ± 0.001	0.188 ± 0.002	-
100	39.17 ± 5.13	0.40 ± 0.08	0.050 ± 0.001	0.100 ± 0.002	0.256 ± 0.019
	24.52 ± 2.65	0.24 ± 0.10	0.052 ± 0.001	0.106 ± 0.002	0.267 ± 0.045
	24.35 ± 3.42	0.24 ± 0.10	0.053 ± 0.001	0.100 ± 0.002	0.278 ± 0.051

of protein–DNA interactions in the process of complex formation. In studies conducted under similar conditions anisotropy equal to 0.26 was reported (Vogel *et al.*, 2002). This value is similar to the fitted final anisotropy in the majority of our experiments.

There are two sites on both promoters, P1 and P2, for specific association with RNAP. They are much closed to each other; a shift is equal to 5 bp and 22 bp for *lac166* and *gal178*, respectively. A simultaneous binding of two RNAP molecules to both sites is impossible (Nisseley *et al.*, 1971; Yu & Reznikoff, 1985; Choy & Adhya, 1993). On the other hand, RNAP can bind to DNA nonspecifically. Hence, two molecules of the enzyme can bind to DNA, but the association constant for one of them should be lower than the other one which describes the specific binding. Indeed, we have observed this discrepancy in the obtained parameters for *gal178* complexes, but not in the case of *lac166*, probably because a free fragment of DNA sufficiently long (70 bp) to bind RNAP nonspecifically is present only in the specific *gal178*–RNAP complex. This confirms the footprinting data which indicate that an 80–90 bp long promoter sequence is protected during DNA–RNAP complex formation (Taniguchi *et al.*, 1979; Taniguchi & de Crombrughe, 1983; Ozoline & Tsyganov, 1995).

The results obtained in the studies of the interactions of both promoter fragments with core RNAP without CRP, were analysed according to model B. Two binding constants equal to 0.67 and $1.9 \times 10^6 \text{ M}^{-1}$ (average values from Tables 3 and 4) were observed for *lac166* and *gal178*, respectively. These low values are typical for nonspecific of enzyme–DNA association, and they are close to the binding constant of the nonspecific interactions equal to $2 \times 10^6 \text{ M}^{-1}$ determined earlier, although that experiment was conducted at a higher ionic strength (deHaseth *et al.*, 1978). The nonspecific association explains well the one-molecule mechanism in case of the *gal178*–core RNAP interaction. As a consequence of this type of interaction, the symmetrical binding of the first molecule to the promoter excluded the binding of the second one.

The specific binding constants in the case of holo RNAP are in the range from 10^6 M^{-1} for weak to 10^{14} M^{-1} for strong promoters. The binding constants measured using kinetic experiments are $1.78 \times 10^6 \text{ M}^{-1}$ for *lac* promoter at 25°C (Liu *et al.*, 2003) and for a stronger promoter, *lacUV5*, $4.4 \times 10^6 \text{ M}^{-1}$ at 30°C (Ross & Gourse, 2005). The binding constant for *lac* and holo RNAP is 10^9 M^{-1} at 4°C (Roy *et al.*, 2004) and $1.45 \times 10^7 \text{ M}^{-1}$ at 37°C (Herbert *et al.*, 1986). The binding constant of the interaction between λP_R promoter and holo RNAP is $2.7 \times 10^7 \text{ M}^{-1}$ at 28°C (Saecker *et al.*, 2002). We obtained values of K_{app} equal to $2.44 \times 10^6 \text{ M}^{-1}$ and $4 \times 10^7 \text{ M}^{-1}$

for *lac166* and *gal178*, respectively (Tables 1 and 2), which are consistent with the results obtained by others, as discussed above.

The K_{app2} of the *gal178*–holo RNAP interaction is equal to $7.7 \times 10^5 \text{ M}^{-1}$, and similar to the binding constant of core RNAP–DNA interactions. Earlier studies of nonspecific interactions of holo RNAP with DNA indicate the value $1 \times 10^5 \text{ M}^{-1}$ (deHaseth *et al.*, 1978). It confirms the hypothesis about a nonspecific interaction of a second enzyme molecule with *gal178*, and the higher value of K_{app} is probably a result of a two-fold lower ionic strength in our experiment than in the earlier one.

The next step of the present studies was conducted in the presence of CRP. As an enhancer of transcription, it inhibits its start from the P1 sites of both promoters, *lac* and *gal* (Nisseley *et al.*, 1971; Yu & Reznikoff, 1985; Choy & Adhya, 1993). The determination of the apparent binding constants allowed us to analyse quantitatively the obtained values at different CRP concentrations and to show an influence of CRP on the specific and nonspecific binding of RNAP to DNA.

The K_{app} of the core RNAP–*lac166* complex was two times higher in experiments with CRP than without it. In the case of the core RNAP–*gal178* complex a new process appeared, i.e. the binding of a second enzyme molecule to the promoter sequence. K_{app1} and K_{app2} measured with CRP are 15 times higher and six times lower than without CRP, respectively. Because of the saturation of the specific sites and in the absence of nonspecific CRP–DNA interactions (which are reasonable, according to experiment conditions), this equilibrium shift should be explained by a specific CRP–DNA interaction.

There are two mechanisms of transcription activation by CRP: protein-induced DNA bending (angle of about 80°) and protein–protein interactions between CRP and RNAP determinants (Lawson *et al.*, 2004). Both of them could also enhance the formation of the core RNAP–DNA–CRP complex. It explains also the two-molecule mechanism of the core RNAP–*gal178* interaction in the presence of CRP. CRP can force asymmetrical binding of core RNAP to DNA. In effect, a long enough DNA fragment is exposed and the next core RNAP molecule is bound to the promoter. The difference of the measured K_{app} for the two nonspecific core RNAP–DNA interactions can result from the different lengths of the promoters fragments. The shorter sequence in the case of the core RNAP–CRP–*gal178* complex has a lower affinity than the whole fragment of DNA.

Similar results were obtained in the experiments with holo RNAP. The studies for *lac166* were conducted with two forms of CRP: its wild type and the T158A variant mutated in AR1. According to the data obtained so far, T158 plays a key role

in class I promoter activation. Its replacement by alanine decreased the transcription activation *in vivo* substantially (Gaal *et al.*, 1996; Niu *et al.*, 1996). Also, the RNAP–CRP interaction, in the absence of the promoter sequence was disturbed by this mutation (Heyduk *et al.*, 1993). Surprisingly, we did not observe any significant differences between CRPwt and its mutant. There are many interactions during the transcription complex formation and the disturbance of only one of them could be too little to be visible. Furthermore, we observed only the formation of the closed complex, and the effect of the mutation could be stronger during the next steps of transcription, like open complex formation.

The K_{app} of the complex formation was two times higher in the presence of CRPwt or its mutant than in their absence. In kinetic experiments conducted under similar conditions, K_{app} increased 3.7- and 1.8-fold in the case of promoters *lac* and *lacP1₁₋₆* (an artificial, stronger promoter), respectively, when experiments without and with CRPwt were compared (Liu *et al.*, 2003; 2004). The difference between the values obtained with the use of kinetic and fluorescence anisotropy methods can be explained by the use of a promoter which excluded polymerase binding to the P2 site in the kinetics studies, in contrast to our studies with an unmodified promoter. Holo RNAP binds preferentially to the *lacP2* site in the absence of CRP *in vitro*, therefore the *P1lac* site is inaccessible (Peterson *et al.*, 1985). On the other hand, promoter-bound CRP is a P2 site repressor (Yu & Reznikoff, 1985). As a result of that, the measured K_{app} in the presence of CRP is compared with two binding processes of RNAP to promoter sites in the absence of CRP. The stronger interaction of holo RNAP with *lacP2* than with *lacP1* explains the low amplitude of the observed change.

The holo RNAP–*gal178*–CRP complex was analysed on the basis of comparison of three CRP variants: CRPwt, CRPH19A and CRPH21A mutants. These amino-acid residues are part of AR2, which interacts with “165” determinant of α NTD. Their replacement with alanine induced a 5-fold decrease of *in vitro* transcription on *CC(-41,5)* promoter (Niu *et al.*, 1996).

The BB model was the best to describe the observed mechanism. In studies using mutants, the measured K_{app1} was, frequently higher than the one determined in the absence of CRP (Table 2). The best dispersion of the residuals was observed for the model in which K_{app1} was of the order of 10^8 – 10^9 M⁻¹ (Fig. 2). A limitation of the number of parameter by using a fixed value of K_{app1} (see Table 2) was necessary to obtain correct parameter values as in the case of the systems with CRPwt. That solution did really improve the fit quality. The necessity of using different ways of analysis points to the

diversity in the behaviour of different CRP forms. The lower K_{app1} values obtained in the presence of the CRP mutants indicate a defect in the ability of CRP to induce an equilibrium shift to the specific complex. Unfortunately, the correlation between the measured K_{app1} and the CRP concentration was poor; therefore we could not calculate the quantitative effect of the CRP presence more precisely. We determined that the presence of CRP induced a 3- to 25-fold increase of the K_{app} of complex formation. This effect was comparable to the one derived from kinetic experiments, where a 4- to 10-fold increase was observed (Herbert *et al.*, 1986; Goodrich & McClure, 1992; Roy *et al.*, 2004). This result is a positive verification of the applied analysis.

According to earlier assumptions, the lower binding constant, K_{app2} , describes nonspecific holo RNAP–DNA interaction. Indeed, its values were insensitive to CRP types and their concentration and they are equal to 10^6 M⁻¹.

Our studies confirm the structural role of CRP in the transcription activation mechanism of *lacP1* and *galP1* promoters. Its presence enhances the affinity of both RNAP forms: core and holo to the promoter sequence by inducing additional interactions. The protein–protein interactions and/or DNA bending induced by CRP change the core RNAP–DNA interaction from nonspecific to specific.

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