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Purification of Core Enzyme of *Escherichia coli* RNA Polymerase by Affinity Chromatography

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Abstract—A method for isolation of a highly purified preparation of *E. coli* RNA polymerase core enzyme was developed based on IMPACT technology and dissociation of the RNA polymerase complex with σ^{70} subunit. Washing of the immobilized RNA polymerase with 5-10 mM solution of glutamate (pH 5.0-5.5) completely removed the σ^{70} subunit from the holoenzyme and decreased amounts of protein admixtures. The possibility of reconstruction of the RNA polymerase holoenzyme directly on the affinity column was demonstrated. Activities of the resulting RNAP core enzyme preparations were tested by *in vitro* transcription. Some amino acids and their mixtures were shown to influence the *in vitro* transcription. The findings indicate that changes in the transcription efficiency in the presence of amino acids should be associated with a specific destruction of the interaction between σ^{70} subunit and the core enzyme.

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Key words: *E. coli* RNA polymerase, subunits, affinity chromatography, IMPACT system, transcription, amino acids

Transcription in bacteria, including *E. coli*, is realized by RNA polymerase (RNAP) [1, 2], the core enzyme of which consists of subunits $\alpha_2\beta\beta'\omega$. The attachment of the σ subunit to the core enzyme results in the so-called holoenzyme capable of specifically recognizing signals of transcription initiation (promoters). In *E. coli* there are a number of σ subunits that are active under different conditions at different stages of cell growth [3-5]. During the exponential growth stage, σ^{70} subunit is the major factor. To detect functionally important regions within this subunit, it is promising to obtain corresponding mutants and to study their features, e.g. the ability to bind with the core enzyme and interact with the promoter DNA within the holoenzyme. However, for such *in vitro* experiments it is necessary to have highly purified preparations of both the core enzyme and the mutant forms of σ subunit. In our previous work we have shown a possibility of rapid and efficient isolation of RNAP preparations with the predominant holoenzyme using the IMPACT system through a heterologous expression of one of the major subunits as "fused" proteins with the intein domain and the chitin-binding domain (CBD) [6]. In the present work we have prepared a highly purified preparation of the RNAP core enzyme completely free of the σ subunit using washings by solutions of free amino acids of the column with the immobilized enzymes.

Abbreviations: CBD, chitin binding domain; IMPACT, intein-mediated purification with affinity chitin-binding tag (a system for affinity purification of proteins); IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; RNAP, *E. coli* RNA polymerase.

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MATERIALS AND METHODS

Reagents used were as follows: Tris and EDTA (Merck, Germany), isopropyl-β-D-thiogalactopyranoside (IPTG) (MP Biomedicals Inc., Germany), polyacrylamide (Fluka, Switzerland), ATP and heparin (Sigma, USA), [γ-^{32}P]ATP (185 PBq/mol) and [α-^{32}P]UTP (148 PBq/mol) (Izotop, Russia), a kit of ribonucleoside triphosphates (Boehringer, Germany), tryptone, agar, and yeast extract (Difco, USA), BSA (SibEnzyme, Russia), chitin beads (NEB, USA), dithiothreitol, ampicillin, and L-amino acids (Serva, Germany).

Enzyme preparations used were as follows: phage T4 polynucleotide kinase (Fermentas, Lithuania), *Thermus aquaticus* DNA polymerase and *Taq*SE polymerase (SibEnzyme, Russia), *E. coli* RNAP core enzyme (Epicenter Technologies; 0.54 μg/μl, 1 U/μl).

Main buffers: TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), transcription buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol), lysis buffer (2× TE-buffer, 500 mM NaCl, 0.05% Triton X-100), buffer A (2× TE-buffer, 100 mM NaCl, 0.05% Triton X-100).

Expression plasmids pC4-b and pC4-c with genes of the β and β′ subunits, respectively, have been described earlier [6]. The plasmid pR-2t containing the consensus promoter and terminator λ*oop* was constructed on the bases of the plasmid pLSR [7] and a small EcoRI-HindIII fragment of the plasmid pKD30 [8].

Polymerase chain reactions (PCR) were performed in a programmed CycloTemp-107 thermostat (Resurs-Pribor, Russia).

A DNA fragment 101 bp in length (RT101) containing a consensus promoter was prepared by a standard PCR method [9] with the plasmid pR-2t as a template and oligonucleotides TACTAGGTCATATTACGAAGCG (22-tr) and AATCCTCGAGGTCGACTC (18-vl) as primers.

The label 5′-^{32}P was introduced into oligonucleotides and DNA fragments using T4 polynucleotide kinase and [γ-^{32}P]ATP by the routine method [10].

Electrophoresis of polynucleotides was performed in 8% denaturing polyacrylamide gel in glass plates (25 × 20 × 0.04 cm) in Tris-borate buffer (pH 8.3) containing 8 M urea at the field strength of 50 V/cm.

Electrophoresis of protein–DNA complexes in 4% nondenaturing polyacrylamide gel was performed in glass plates (25 × 20 × 0.08 cm) in Tris-borate buffer (pH 8.3) at the field strength of 20 V/cm.

Protein preparations were analyzed by the Laemmli method [11] in 8% denaturing SDS-polyacrylamide gels. The gels were stained with PageBlue™ Protein Staining Solution (Fermentas).

Radioautographs were obtained with a PhosphorImager (Molecular Dynamics, USA). Radioactive zones were counted using the ImageQuant v.5.0.1 program.

The protein preparations were concentrated on Microcon® centrifugal ultrafiltration cells (Millipore, USA).

Escherichia coli cells were cultured on medium dyt consisting of tryptone (16 g), yeast extract (10 g), and NaCl (5 g) per liter. Transformation, cloning, isolation of the plasmid DNA, purification of DNA fragments in gels, and also other procedures of gene engineering were performed by standard methods [10].

Isolation of RNAP preparations. To 50 ml of the medium dyt supplemented with ampicillin (100 μg/ml), 2 ml of overnight culture of *E. coli* ER1821 with plasmids pC4-b or pC4-c was added, the culture was grown for 1-1.5 h on a roller at 35°C to absorption A_{600} = 0.6-0.8, supplemented with IPTG (2 mM), and grown for 2 h more. The cells were precipitated by centrifugation, the supernatant was removed, the precipitate was suspended in 0.8 ml of lysis buffer, and the cells were destroyed by ultrasonication. The resulting cell homogenate was centrifuged at 12,500*g* for 15 min at 4°C. The cleared homogenate was passed through a column with 0.2 ml of chitin beads. The column was washed thrice with 0.2 ml of lysis buffer, thrice with 0.2 ml of buffer A, and thrice with 0.2 ml of buffer A containing the ligand under study (2.5-25 mM), then incubated for 30 min at 37°C, and after the incubation washed twice with 0.2 ml of the same solution, thrice with 0.2 ml of buffer A, and rapidly with 0.23 ml of buffer A containing 50 mM dithiothreitol; then 0.07 ml of the same buffer was added and left for 16-20 h at 4°C. The detached proteins were eluted from the column thrice with 0.2 ml of buffer A. Experiments were performed with amino acids Glu, Phe, Leu, Lys, Arg, Gly, Asp and mixtures (Phe + Leu, Phe + Leu + Lys, Phe + Leu + Gly, etc.) and also in the absence of amino acids (control). The resulting preparations were analyzed by SDS-PAGE. The specific activity of the preparations determined as described in [12] with DNA of calf thymus and plasmid DNA used as templates was 1.5-2.5 U/μg.

All protein preparations were concentrated by ultrafiltration, transferred into buffer A, and stored at –20°C in buffer A with glycerol (50%).

The σ^{70} subunit was isolated by affinity chromatography using the IMPACT technology as described in work [6].

Saturation of RNAP with σ subunit. RNAP preparations (50-100 nM) were incubated for 30 min at 30°C in transcription buffer with sixfold molar excess of σ subunit. At the solid-phase saturation of the enzyme with σ subunit, the column with immobilized RNAP was washed with 300 μl of σ subunit solution (0.1-0.2 mg/ml) in buffer A.

Study on production of RNAP complexes with DNA. The ^{32}P-labeled PCR-fragment RT101 (5-10 nM) containing a consensus promoter was incubated for 15 min at 37°C with RNAP preparation under study (50 nM) in 10 μl of transcription buffer, and then 1 μl of 50% glycerol with marker dyes xylene cyanole (0.01%) and Bromophenol Blue (0.01%) and heparin (final concentration 100 μg/ml) were added. The mixture was placed into a cell of 4% nondenaturing polyacrylamide gel and electrophoresed at room temperature at 20 V/cm; then the gel was dried and radioautographed.

Transcription *in vitro*. RNAP preparations (10-100 nM) were incubated with a DNA-template (10-100 nM) for 10 min at 37°C in 6 μl of transcription buffer, then they were supplemented with 2 μl of the same buffer containing mixture of labeled ribonucleoside triphosphates (final concentrations: 0.1 mM ATP, GTP, and

CTP; 0.05 mM UTP; 10 kBq [α-^{32}P]UTP) and heparin (final concentration 100 μg/ml). The mixture was incubated for 10 min at 37°C, and then the transcription reaction was stopped by addition of 6 μl of "stop-solution" (0.01% xylene cyanole, 0.01% Bromophenol Blue, and 25 mM EDTA in 80% formamide). The mixture was placed into a cell of 8% denaturing polyacrylamide gel, electrophoresis was performed, the gel was dried, and radioautographed. To study the effects of amino acids, they were added into the reaction mixture to the final concentration of 5-10 mM at different stages: before addition of DNA, before addition of the nucleoside triphosphate mixture, or concurrently with triphosphates. All experiments were performed not less than thrice, and the experiment error was not higher than 12%.

RESULTS AND DISCUSSION

The RNAP core enzyme is usually obtained as a result of separation by chromatography of the mixtures of the holo- and core enzymes. In particular, a very expensive chromatography on a Mono-Q column is used [13]. In the previous work [6] we developed a method of rapid and highly efficient isolation of RNAP preparations on a chitin-containing column using heterologous expression of one of the core enzyme major subunits (α, β, or β′) as a protein with an intein and chitin-binding fragments. The resulting enzyme is essentially presented as the holoenzyme. However, in RNAP preparations isolated with involvement of the modified β subunit the content of σ subunit is significantly lower (15-20%). It seems that the intein-CBD module attached to the C-terminal of β subunit prevents the interaction of σ subunit with the core enzyme stronger than in the case of the modified α and β′ subunits; therefore, just this variant of isolation with involvement of the modified β subunit seems to us the most promising for further improvement of the method for preparing the pure core enzyme.

As the main strategy, additional washings of the chitin-containing column with immobilized RNAP were performed using reagents promoting the dissociation of σ subunit. To assess the efficiency of such washings, RNAP preparations were detached by a standard treatment with dithiothreitol and analyzed in SDS-PAGE (Fig. 1, lane *1*). Gel staining with Coomassie did not allow us to detect small (residual) amounts of σ subunit in the isolated proteins; therefore, the presence of σ subunit was assessed by activity of RNAP preparations in the *in vitro* reaction of one-round transcription in the presence of heparin immediately after their isolation and also upon the additional saturation with σ subunit. The experiments were performed in the presence of three-fivefold excess of a template DNA. As a template the supercoiled plasmid DNA (pR-2t) was used, which contained a strong constitutive consensus promoter located before the transcription terminator *λoop* and generating a transcript of 167 nucleotides in length. The ratio of transcription products isolated without the additional saturation of RNAP preparations with σ subunit and under conditions of its excess allowed us to assess the content of σ subunit in the resulting preparations.

First of all, we tried to remove σ subunit using salt solutions of different concentration, in particular, with 0.5-1.0 M NaCl [14], heparin (5 and 50 μg/ml), and also with chaotropic agents (Triton X-100, etc.). In some cases this allowed us to markedly decrease (to 5-10%) the

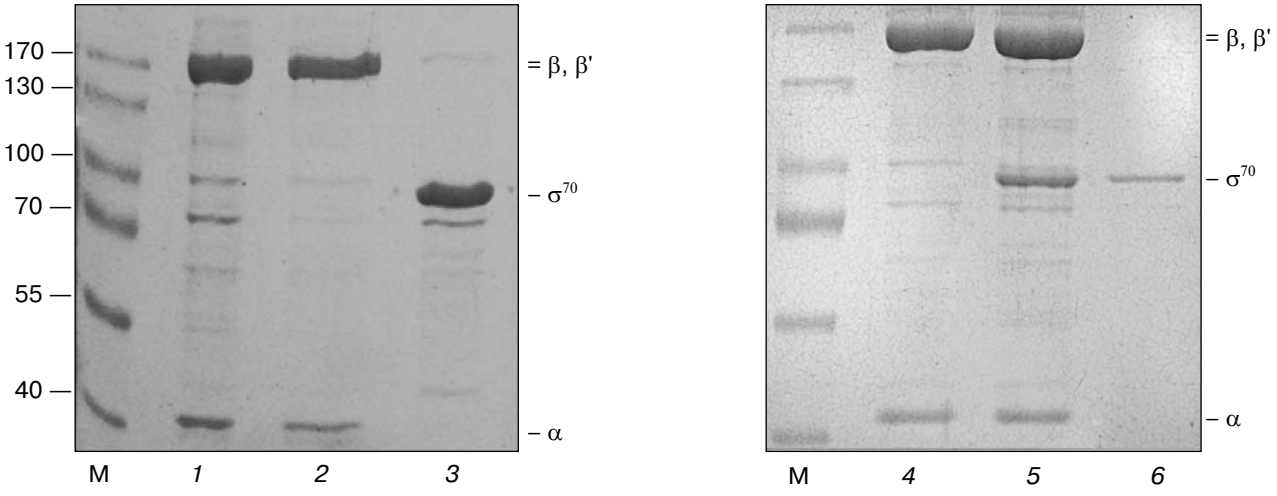

Fig. 1. Electrophoresis in 8% SDS-polyacrylamide gel of RNAP preparations isolated from cells with plasmid pC4-b by the standard IMPACT method (*1*, *4*), with additional washing of the column with 5 mM solution of glutamic acid (*2*) or with a solution containing σ subunit (*5*). Lanes: M, marker proteins; *3*, *6*) σ subunit. To the right positions of RNAP subunits are shown; the numbers to the left show molecular weights of marker proteins (kDa).

content of σ subunit in the final preparations but not to remove it completely. Therefore, we took into account an observation of Maitra et al. [15] that at relatively low concentrations (2.5-10 mM) of mixture of hydrophobic amino acids leucine and phenylalanine the RNAP holoenzyme dissociated into the core enzyme and σ subunit. The authors suggested that these amino acids could specifically affect the intersubunit contacts (between σ subunit and the core enzyme). However, the use of this mixture of amino acids did not completely remove the σ subunit, but it markedly decreased its content (to less than 5%). Possibly the other authors' success was associated with specific features of the system they used where a histidine-containing fragment necessary for the enzyme immobilization on Ni-agarose was located inside the protein molecule of σ subunit near the region 3.1 that could additionally weaken the interaction with the core enzyme. Therefore, we tried to find amino acids (or their mixtures) promising for removal of σ subunit in our case.

According to the literature, some regions within σ subunit are involved in the interaction with the core enzyme, and the interaction character changes depending on the stage of the transcription initiation [16-20]. In particular, the strongest contacts exist between regions 2.1-2.2 (373-416) and the so-called "coiled-coil" domain of the β′ subunit (260-309) and also between regions 4.1-4.2 and the domain "flap" of the β subunit (885-914) [17, 21-23]. Structures of the above-mentioned regions include conservative residues among which are R275, E295, A302 (β′ subunit), L384, L402, D403, Q406, E407, E555, F563, L598, L607 (σ subunit), L901, L902, I905, F906 (β subunit). Involvement of these residues in the most important inter-subunit contacts was shown using site-specific mutagenesis and various biochemical approaches [24]. Thus, the substitutions R275Q, E295K, and A302D (β′ subunit) lead to inability of the RNAP core enzyme to bind σ subunit under *in vitro* conditions [25]; point substitutions L384A, V387A, L402F, D403A, Q406A, E407K/A, E555K/A, R562A, L598A, R541C, and L607P in σ subunit significantly decrease (more than 5-15-fold) its affinity for the core enzyme [26]; mutations L384A and E407K are shown to promote the transition of RNAP to the elongation stage due to weakening of the σ subunit interaction with the core enzyme [27, 28]. There are data on the immediate contact of Q406 and E407 with the "coiled-coil" domain of (β′ subunit), and these residues are exposed on the protein surface [17]. The model of σ subunit interaction with the core enzyme with involvement of the above-mentioned residues was proposed in work [24].

Influence of free amino acids on transcription is virtually unknown. In solutions amino acids are able to compete with the corresponding amino acid residues within protein structures and thus to weaken interactions between proteins via the side radicals. It was recently shown [29, 30] that glutamate ion as a neutral salt could regulate transcription both *in vitro* and *in vivo*, under conditions of osmotic shock. The mechanism of the glutamate effect is still unknown, but it is supposed to influence just the interaction between σ subunits (σ^{70} and σ^{38}) and the core enzyme and thus to regulate the transcription from the corresponding promoters. In an early work by Krakow [31], copolymers poly(Glu, Tyr), poly(Glu, Phe), and poly(Glu, Trp) were shown to inhibit RNAP of the bacterium *Azotobacter vinelandii* and also to prevent its aggregation.

To find amino acids capable of promoting the dissociation of σ subunit from the holoenzyme, we performed *in vitro* transcription experiments that were different in the stage of addition of various amino acids into the reaction mixture. The first series of experiments included a preincubation of the RNAP holoenzyme isolated by means of the IMPACT system with amino acids before the standard reaction of the one-round transcription. In the second series amino acids were added to the preformed open complex RNAP–DNA and the mixture was incubated for the same time as in the first series until the transcription with the nucleoside triphosphate mixture was initiated; in the third series amino acids were added concurrently with nucleoside triphosphates upon the formation of the open complex RNAP–DNA. The concentration of each of the amino acid in the reaction mixture was varied in the range of 5-10 mM. Considering the literature data on interprotein contacts, different amino acids (acidic, basic, neutral) were chosen for the studies. Figure 2 exemplifies results of some experiments performed with individual amino acids (Phe, Leu, Lys, Glu, Gly) or with the mixture Phe + Leu. The transcription efficiency was significantly decreased in the case of amino acids Phe and Leu and also of their mixtures if RNAP was preincubated with them before the addition of promoter DNA. However, incubation with amino acids of the preformed complex RNAP–promoter did not decrease the yield of the full-size transcript, but even increased it (by 20-50%). These findings indicate that the amino acids studied influence the stages preceding the formation of the open complex, namely, disturb the interaction between σ subunit and the core enzyme in the case of free RNAP, or prevent the primary recognition by the holoenzyme of the promoter region of DNA. The more pronounced effect of amino acid mixtures suggests an additivity of their effects, and it seems that the transcription is inhibited due to "displacement" of the residues Phe and Leu participating in the macromolecular interactions by the corresponding free amino acids. During the stage of transcription initiation (triphosphate addition) the presence of amino acids is favorable for the enzyme transition to the elongation stage (an increase in the amount of the full-size product due to the RNAP exit from the stage of abortive synthesis), most likely due to weakening of interactions between σ subunit and RNAP. Note that basic amino acids Lys and Arg did not significantly influence

the *in vitro* transcription in the case of preincubation with RNAP but also promoted the transition to the elongation stage (Fig. 2). Addition of Gly into reaction mixtures caused virtually no change in the transcription character. Similar data (not presented) were obtained for alanine, glutamine, and neutral glutamate. The picture was more complicated in the case of glutamic acid: its addition slightly shifted the pH of the medium. This shift depended on the amino acid concentration and was likely to influence the catalytic activity of RNAP, decreasing the transcription level in all three series of experiments. However, the effect was most pronounced on the incubation of amino acids before the open complex formation (Fig. 2). These findings correlate with the literature data on the role of amino acid residues Leu, Phe, and Glu in the establishment of contacts between the core enzyme and σ subunit.

These findings were used for development of the method for isolation of the RNAP core enzyme in the IMPACT system. In particular, for washing of the affinity column with the immobilized RNAP we tested some individual amino acids: "neutral" Phe, Leu, Gly, and Gln, "acidic" Glu and Asp, and "basic" Lys and Arg, as well as different mixtures, e.g. Phe + Leu, Phe + Leu + Gly, Phe + Leu + Lys, and Phe + Leu + Gln. The total concentration of amino acids was varied in the range of

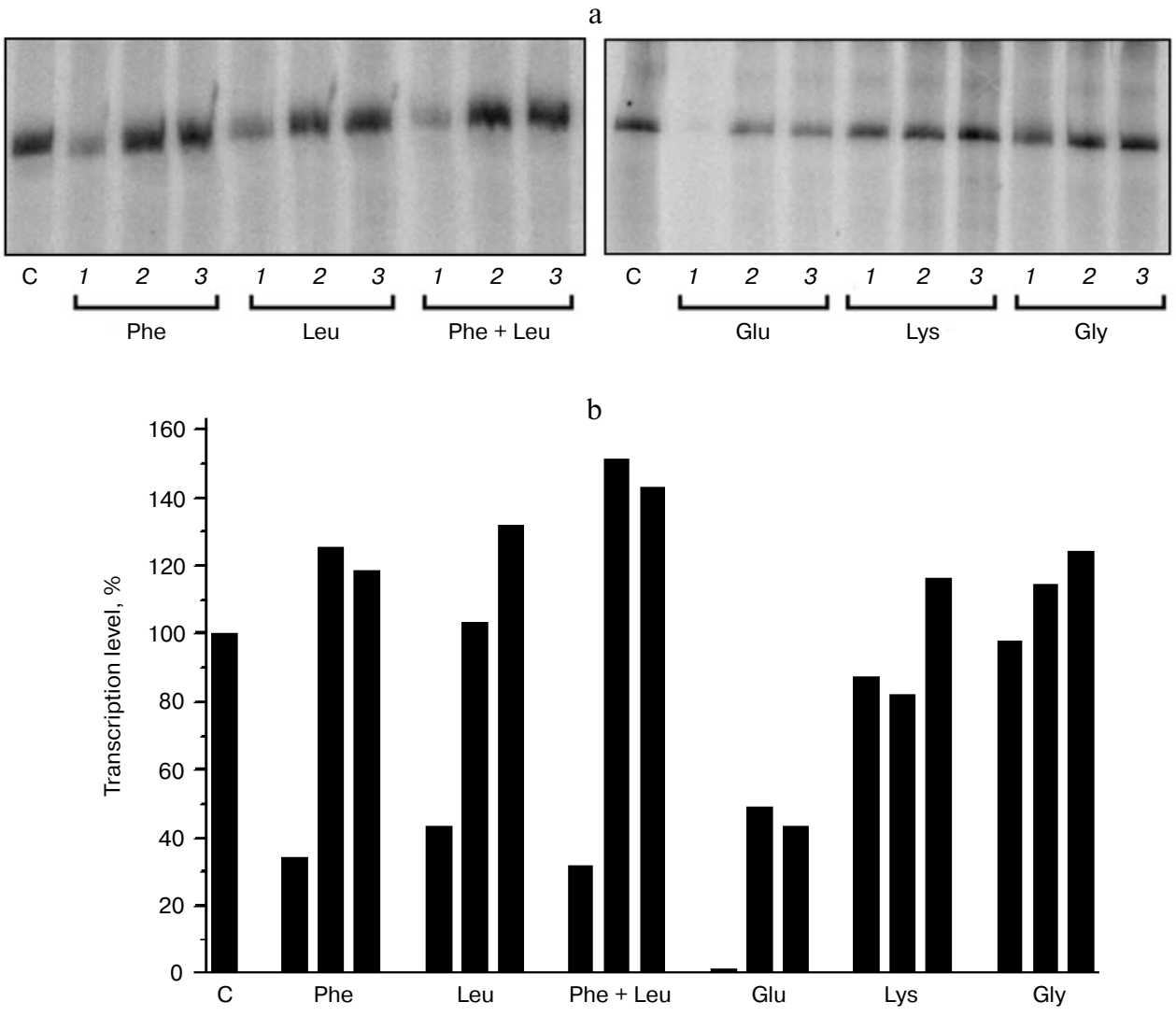

Fig. 2. Effects of free amino acids on *in vitro* transcription. a) Radioautograph of the electrophoretic separation in 8% denaturing polyacrylamide gel of the one-round transcription products on the promoter-containing plasmid DNA pR-2t using the RNAP preparation isolated by the standard IMPACT technology in the absence of free amino acids (lane C) or in the presence of amino acids (indicated below). Lanes: *1*) RNAP preparation was incubated with amino acids for 15 min before the addition of DNA; *2*) amino acids were added to the previously formed complex of RNAP–DNA and incubated for 15 min before the initiation of transcription by nucleoside triphosphates; *3*) amino acids were added to the complex RNAP–DNA concurrently with nucleoside triphosphates. The concentration of DNA was 10 nM, of RNAP 50 nM. b) The transcription level in experiments presented in the electrophoregram (a) relative to the transcription level in the experiment taken as 100% on saturation of the control RNAP preparation with σ subunit (lane C in (a)).

2.5-50 mM. In some cases (e.g. Phe + Leu and Phe + Leu + Gln mixtures) the σ subunit content in the final preparations of RNAP was markedly decreased. However, we obtained the best results with solutions of glutamic acid. Thus, incubation of chitin beads with immobilized RNAP in solution of 5-10 mM Glu in transcription buffer (pH 5.0-5.5) for 30 min at 37°C and subsequent washing with the same solution allowed us to obtain preparations of the core enzyme completely free of σ subunit (Fig. 1, lane *2*). Such a preparation was isolated, and its activity was tested in some systems. The absence of RNA products in the one-round transcription using this preparation (Fig. 3, a and b) and appearance of such products upon the addition of σ subunit to the enzyme indicated that RNAP isolated by the additional washing with glutamic acid solution was the pure enzyme. This was also confirmed by data on the formation of the complex with promoter-containing DNA in the presence of heparin (Fig. 3, c and d). The isolated RNAP preparation was unable to form heparin-resistant complexes with the promoter-containing fragment of DNA but acquired this ability upon the addition of σ subunit. For comparison, in Fig. 3 it is shown that washing with other solutions (e.g. 0.5 M NaCl or mixture of 5 mM Phe and Leu) does not completely removed σ subunit. These findings additionally confirm that glutamic acid residues (e.g. E407) are involved in the establishment of the most important contacts of the core enzyme and σ subunit. Note that the

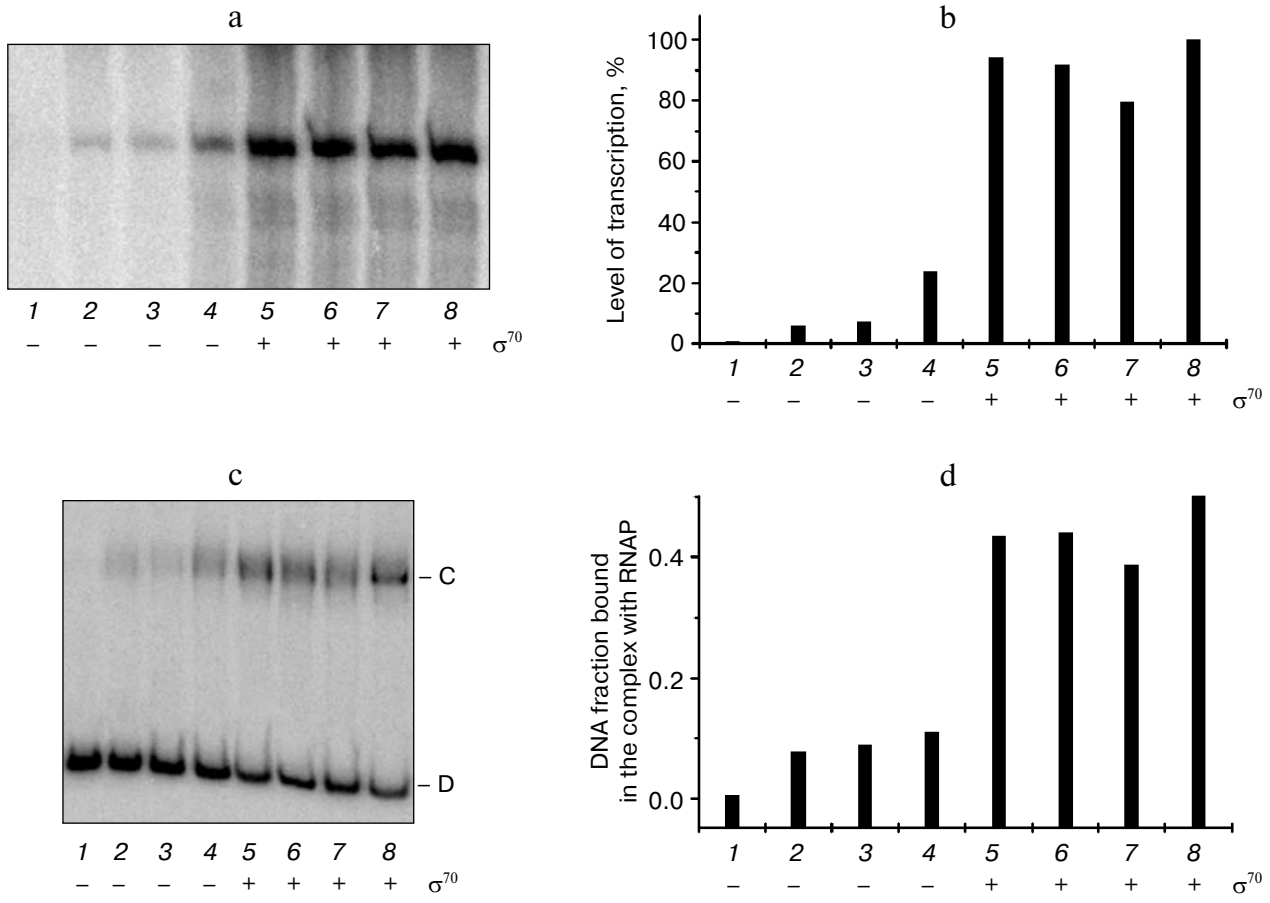

Fig. 3. Effects of washings of the affinity column with different reagents on the content of σ subunit in the isolated RNAP preparations. a) Radioautograph of the electrophoretic separation in 8% denaturing polyacrylamide gel of the one-round *in vitro* transcription products on the plasmid DNA pR-2t with the consensus promoter. The reactions were performed using RNAP preparations isolated by affinity chromatography on a chitin-containing column by the standard method (lanes *4* and *8*) and using additional washings of the column with 5 mM Glu (lanes *1* and *5*), the mixture of 5 mM Phe and Leu (lanes *2* and *6*), or with 0.5 M NaCl (lanes *3* and *7*) after the saturation with σ subunit (+) or in its absence (–). The DNA concentration was 30 nM, and that of RNAP was 10 nM. b) The transcription level in experiments presented in the electrophoregram (a) relative to the level taken as 100% on saturation of the control RNAP preparation with σ subunit (lane *8* in (a)). c) Radioautograph of the electrophoretic separation in 4% nondenaturing polyacrylamide gel of complexes formed by DNA PCR fragment containing the consensus promoter with RNAP preparations isolated by affinity chromatography on a chitin-containing column by the standard method (lanes *4* and *8*) or using additional washings of the column with 5 mM Glu (lanes *1* and *5*), mixture of 5 mM Phe and Leu (lanes *2* and *6*), or with 0.5 M NaCl (lanes *3* and *7*) after saturation with σ subunit (+) or in its absence (–). The arrows indicate positions of the duplex (D) and its complexes with RNAP (C). The concentration of DNA was 10 nM, and that of RNAP was 50 nM. d) DNA fraction bound in the complex with RNAP in experiments presented in the electrophoregram (c).

treatment of immobilized RNAP with amino acids did not cause a decrease in the activity of the enzyme and dissociation of the core enzyme. The specific activity of the RNAP preparation isolated by this method was comparable with the specific activity of commercial preparations from Epicenter Technologies (1.5-2.5 U/μg).

Advantages of the IMPACT technology are rather mild conditions of the affinity chromatography, which provide for retention of the native structure of the isolated proteins. However, in the case of RNAP this results in rather noticeable admixtures of other RNAP-associated proteins in the enzyme preparations isolated by the standard method, especially when the intein-CBD-domain is attached to β′ subunit. Some of these admixtures are involved in one or other stage of transcription [32, 33]. In the present study our attention was focused on the most noticeable admixtures with molecular weight close to that of σ subunit (Fig. 4) because in some cases electrophoretic analysis of the isolated RNAP preparations was associated with difficulties in identification of the band corresponding to σ subunit. We performed special experiments with addition of increasing amounts of σ subunit to aliquots of the same RNAP preparation, and this allowed us to accurately determine its location in the electrophoregram (Fig. 4). Two satellite bands with weights closest to that of σ subunit were isolated from the protein gel, and their analysis by mass spectrometry revealed that the band migrating more rapidly than σ subunit was the protein DnaK (~69 kDa) and the slightly less mobile band was polynucleotide phosphorylase (pnp, 711 kDa) involved in the hydrolysis of mRNA and capable of associating with α subunit, as it was shown for other bacterial RNAPs [34]. We found that washings with different amino acids including hydrophobic ones, glutamic acid, and also with different sets of amino acids markedly decreased contents of all the above-mentioned protein admixtures (compare lanes *1* and *2*, Fig. 1). This phenomenon seems to be universal, and free amino acids of different nature can be used for purification of desired proteins from weakly associated impurities. It was shown in the work [35] that the washing of the Ni-agarose column with immobilized His_6-tagged RNAP with glycine solution removed some protein admixtures.

Our approach has some advantages as compared to the most efficient earlier described methods of core enzyme preparation by *in vitro* reconstruction from subunits [36-38] or by isolation using a Mono-Q column [13, 39, 40]. In our case RNAP was assembled *in vivo* under native conditions, the core enzyme was isolated by affinity and purified by a simple treatment of the column with reagents not affecting the protein structure, and pure highly active preparations of the core enzyme were obtained. This technology can be used for isolation of both natural RNAP and the mutant core enzyme.

We have also demonstrated the possibility of isolation of RNAP holoenzyme using the additional washing

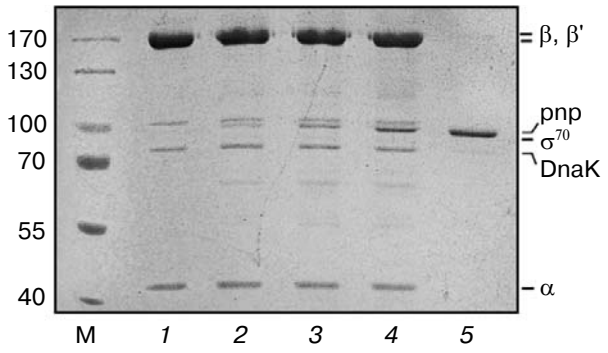

Fig. 4. Electrophoresis in 8% SDS-polyacrylamide gel of RNAP preparations (2 μg) isolated from cells with plasmid pC4-b by the standard IMPACT method without addition of σ subunit (*1*) and with addition of σ subunit: 0.05 (*2*), 0.25 (*3*), and 0.5 μg (*4*). Lanes: M, marker proteins; *5*) σ subunit. To the right positions of RNAP subunits and protein admixtures (pnp and DnaK) are shown; to the left molecular weights of protein markers (kDa) are shown.

of the affinity column with a solution containing σ subunit (Fig. 1, lane *5*). This scheme results in easy isolation of modified variants of the holoenzyme using σ^{70} subunit mutants or alternative σ factors.

Thus, in the present work some free amino acids are shown to specifically influence the *in vitro* transcription catalyzed by *E. coli* RNA polymerase. The findings confirm that amino acids promote the weakening of the interaction between the core enzyme and σ subunit, which under certain conditions results in dissociation of the latter from the holoenzyme. Moreover, the findings indirectly prove that residues Phe, Leu, and Glu are important for interaction between the core enzyme and σ subunit. The findings allowed us to develop a simple and highly efficient method of affinity isolation of the core enzyme of RNA polymerase using the IMPACT system supplemented by additional washings of the columns containing the immobilized enzyme with solutions of free amino acids, which induced dissociation of the residual σ subunit. Highly active pure preparations of *E. coli* RNAP core and holoenzyme have been isolated.

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