Uncoupling of Allosteric and Oligomeric Regulation in a Functional Hybrid Enzyme Constructed from *Escherichia coli* and Human Ribonucleotide Reductase*

Yuan Fu,[†] Marcus J. C. Long,[‡] Mike Rigney,[§] Saba Parvez,[†] William A. Blessing,[†] and Yimon Aye[,†,¶]*

SUPPORTING INFORMATION

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SUPPORTING METHODS

Recombinant Expression of HE and Single Domains of RNR-α. BL21-CodonPlus(DE3)-RIL competent cells (Stratagene) were transformed with pet28a–His_6-HE, or -H-NTD. For E-NTD and E-CB, non-codon enhanced BL21 competent cells were used. Cells were then grown overnight on LB agar plates in the presence of 50 μg/mL kanamycin. In a typical expression procedure, a 5-ml culture containing 50 μg/mL kanamycin (and 50 μg/mL chloramphenicol for CodonPlus cells) was inoculated with a single colony and incubated overnight at 37 ºC. The overnight culture was subsequently diluted into 1.2 L LB media containing 50 μg/mL kanamycin. Cells were grown to OD_{600} of 0.6–0.8 at 37 ºC and induced with 250 μM IPTG for 16 h at 28 ºC for HE. Recombinant expression of NTD alone of H-α and E-α, and CB alone of E-α was attempted 16 ºC. The cells were harvested by centrifugation (7,000 x g for 20 min), flash-frozen in liquid N_2, stored at −80 ºC and used within two weeks for protein isolation. Approximately 3 g of wet cell paste was harvested per L of culture.

Isolation of HE and E-α-CB (Figure 2A and S1B). All steps were carried out at 4 ºC. The cell pellet was suspended in the lysis buffer (5 vol/g) containing 50 mM NaH_2PO_4 (pH 7.0), 0.1% Triton X-100, 10 mM imidazole and 5 mM BME with Roche protease cocktail inhibitors. The cells were lysed by two passages through a French pressure cell operating at 13,000 psi and cell debris was removed by centrifugation (20,000 x g, 30 min). DNA was precipitated by dropwise addition of streptomycin sulfate (2% wt/vol) with continuous stirring over 15 min, with an additional 15 min incubation. The precipitated DNA was removed by centrifugation (20,000 x g, 30 min) and supernatant

was incubated for 1 h with TALON resin (1 mL bed vol/g cell pellet) that had been pre-equilibrated with lysis buffer. Subsequent to removal of flow-through, the column was washed under gravity with the wash buffer containing 50 mM NaH_2PO_4 (pH 7.0), 0.1% Triton X-100, 50 mM imidazole, 5 mM BME and 800 mM NaCl until the A_{280} absorbance reaches background level. The bound His_6-tagged protein was released with elution buffer containing 50 mM NaH_2PO_4 (pH 7.0), 125 mM imidazole, 1 mM BME and 300 mM NaCl. The fractions with eluted proteins were identified by A_{280} absorbance, pooled and concentrated using Amicon Ultra (Millipore, 30,000 MWCO). Removal of imidazole and exchange into storage buffer [50 mM Tris-Cl (pH 7.6), 100 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, and 5% glycerol] was carried out using Sephadex G25 chromatography (GE healthcare). Typical yields for HE was 4–5 mg/10 g of wet pellet, and for E-CB, 10 mg/10 g.

Pull-down of E-NTD, H-NTD and HE (Figure S1B). All steps were performed at 4 ºC. Buffers used were identical to protein isolation buffers. Lysis was carried out as described above using 3 g of each of the three different pellets. In each case, 5% of the homogenous lysate solution was incubated with 50 µL bed vol of TALON resin for 40 min. Subsequent to centrifugation (500 x g, 5 min) and removal of the supernatant, the resin was washed twice (2 x 500 µL) with wash buffer. In between each wash, the resin was incubated with wash buffer for 10 min prior to centrifugation (500 x g, 5 min). Elution of his-tagged protein/domain from the resin was induced by incubating the resin with elution buffer (50 µL) for 15 min followed by centrifugation (4000 x g, 2 min).

Competition Assays (Figure S2B). The reaction mixture contained in a total volume of 30 μL: 50 mM Hepes (pH 7.6), 15 mM $MgCl_2$, 3 mM ATP, 2 mM NADPH, 0.1 mM Trx, 1 μM TrxR, 0.5 μM E-β, 0.5 mM [5-^{3}H]-CDP and either 0, 0.5, 1, 2.5, 5, 7.5, 10 or 15 μM E-CB. The reaction mixture was pre-incubated at 37 ºC for 1 minute prior to initiation with 0.5 μM final concentration of E-α. The reaction was quenched at 5 minutes by the addition of 30 μL of 2% $HClO_4$ and subsequently neutralized with 30 μL of 0.4 M NaOH. [5-^{3}H]-dCDP produced was analyzed as described elsewhere in the manuscript.

SUPPORTING TABLE

TABLE S1. Primers used for the construction of pET-28a –HE and E-CB. (E = *E. coli*, CB = catalytic body)

A) Primers to clone out NTD of H-α

Fwd: ACTTGCACAAAGAAACAAAGGGCCAGTTTGAGCCGCCTGC
Rev: TTGTTAGCAGCCGGATCTCATCAGATCTTACATGCGCCGCT
Fwd extender: CCTGACTATGCTATCCTGGCAGCCAGGATCGCTGTCTCTAACTTGCACAAAGAAACAAAG
Rev extender: CGGATTATCAGTATCTCGCCGCGCGCCTGGCGATCTTCCACCTGCGTAAAAAAGCCTACG

B) Primers to clone out CB of E-α

Fwd: TGGTGCCTCGTGGTAGCCATATGCATGTGATCAAGCGAGATG
Rev: ACAGCGCAGGCGGCTCAAACTGGCCCTTTGTTTCTTTGTGCAAGTTAGAGAC
Fwd extender: ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCTCGTGGTAGCCAT
Rev extender: TTTGCCCATCTCGACCATTTTCACCACGTGGTCGTACAGCGCAGGCGGCTCAAACTGGCC

C) Primers to construct pET-28a-E-CB

E cat. body Fwd: TGGTGCCTCGTGGTAGCCATGGCCAGTTTGAGCCGCCT
E Rev: CTCAGCTTCCTTTCGGGCTTTGTTATCAGATCTTACATGCGCCGCTT

SUPPLEMENTAL FIGURES

FIGURE S1.

A

MGSSHHHHHHSSGLVPRGSHMHVIKRDGRQERVMFDKITSRIQKLCYGLNMDFVDPA
QITMKVIQGLYSGVTTVELDTLAAETAATLTTKHPDYAILAARIAVSNLHKETKGQFEPPA
LYDHVVKMVEMGKYDNHLLEDYTEEEFKQMDTFIDHDRDMTFSYAAVKQLEGKYLVQN
RVTGEIYESAQFLYILVAACLFSNYPRETRLQYVKRFYDAVSTFKISLPTPIMSGVRTPTR
QFSSCVLIECGDSLDSINATSSAIVKYVSQRAGIGINAGRIRALGSPIRGGEAFHTGCIPFY
KHFQTAVKSCSQGGVRGGAATLFYPMWHLEVESLLVLKNNRGVEGNRVRHMDYGVQI
NKLMYTRLLKGEDITLFSPSDVPGLYDAFFADQEEFERLYTKYEKDDSIRKQRVKAVELF
SLMMQERASTGRIYIQNVDHCNTHSPFDPAIAPVRQSNLCLEIALPTKPLNDVNDENGEI
ALCTLSAFNLGAINNLDELEELAILAVRALDALLDYQDYPIPAAKRGAMGRRTLGIGVINF
AYYLAKHGKRYSDGSANNLTHKTFEAIQYYLLKASNELAKEQGACPWFNETTYAKGILPI
DTYKKDLDTIANEPLHYDWEALRESIKTHGLRNSTLSALMPSETSSQISNATNGIEPPRG
YVSIKASKDGILRQVVPDYEHLHDAYELLWEMPGNDGYLQLVGIMQKFIDQSISANTNY
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ACKI

B

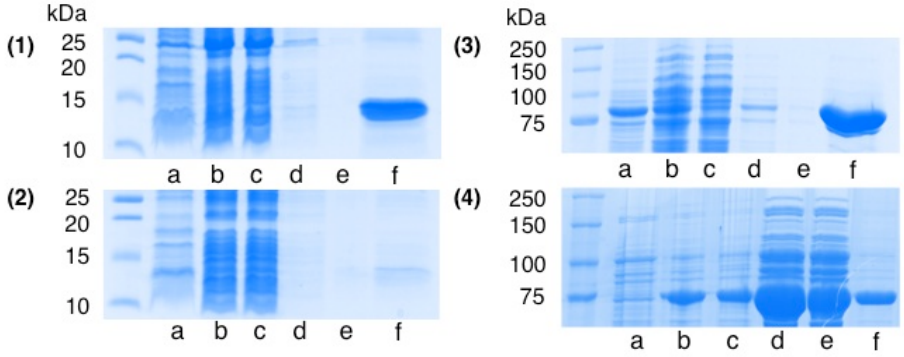
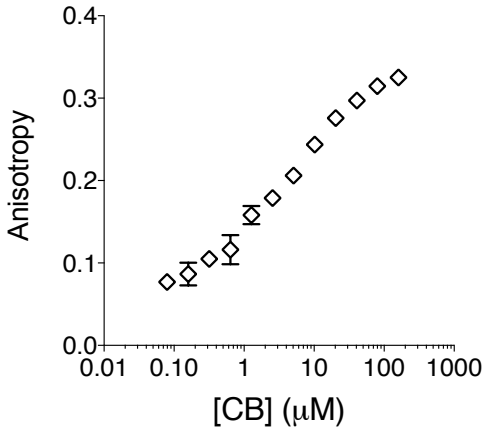


Figure S1. (A) Amino acid sequence of Hybrid His_6-HE. **dark**, His_6-tag and linker bearing thrombin cleavage site; **red**, human N-terminal domain; **blue**, *E. coli* catalytic barrel; **green**, *E. coli* C-terminal tail. (**Blue**+**green** = *E. coli* catalytic body, E-CB). (B) Pull-down analysis validating expression and solubility of His_6-H-NTD (11 kDa) (**1**) and His_6-E-NTD (10 kDa) (**2**), in comparison with His_6-HE (87 kDa) (**3**) and His_6-E-CB (75 kDa) (**4**). Lanes a→f, pellet, lysate, supernatant subsequent to TALON incubation, 1st wash, 2nd wash, elution.

FIGURE S2.

A



B

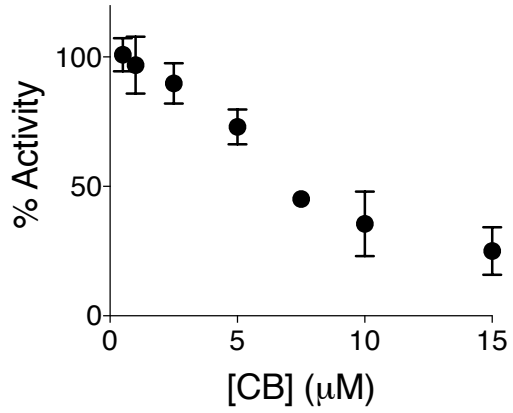


Figure S2. (A) *E. coli* CB was able to bind ligand. Fluorescence anisotropy resulting from titration of 0.1–160 μM *E. coli* CB and 0.5 μM Texas Red-5-dATP. Error range derived from N = 2. Estimated binding affinity of Texas Red-5-dATP to S site within E-CB = 5.3 ± 0.3 μM. (B) *E. coli* CB was able to competitively bind E-β. Catalytic activity of 1:1 E-α: E-β (0.5 μM) was increasingly perturbed with increasing concentrations of E-CB. Calculated IC_{50} = 7.9 ± 1.4 μM. 100% corresponds to specific activity of 515 ± 19 U/mg (calculated per mg of E-α) (0.746 ± 0.028 s^{-1}). Error range derived from at least N = 2.

FIGURE S3.

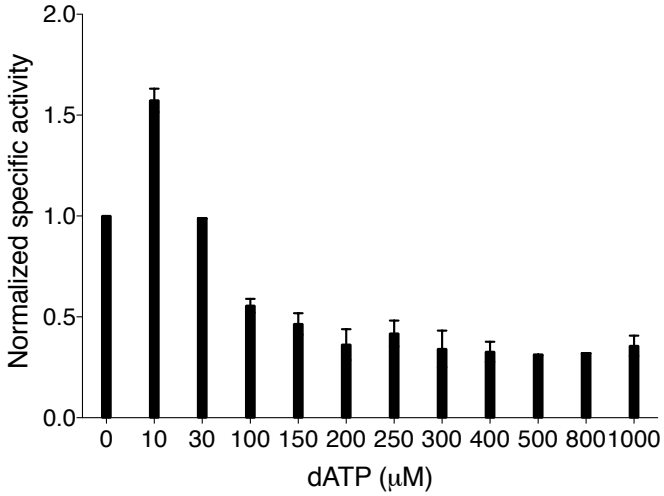


Figure S3. Titration of dATP to canonical H-α. (Also see reference 19 in the manuscript). As in HE (Figure 3A), allosteric activity promotion was observed at low [dATP] through binding to the S site of H-α. However, saturating amount of dATP (A-site binding) led to >70% loss of H-α activity (EC_{50} = 33±7 μM), with respect to the value measured in the absence of dATP. [This is in stark contrast to HE (Figure 3A) wherein HE activities in the absence as well as in saturating amount of dATP remain similar]. Normalized activity of 1.0 on the Y-axis corresponds to 67 ± 8 $nmolmin^{-1}$ of [5-^{3}H]-dCDP produced per mg of H-α. (Note that assays contained no allosteric promoter ATP). Error range was derived from N = 2.

FIGURE S4.

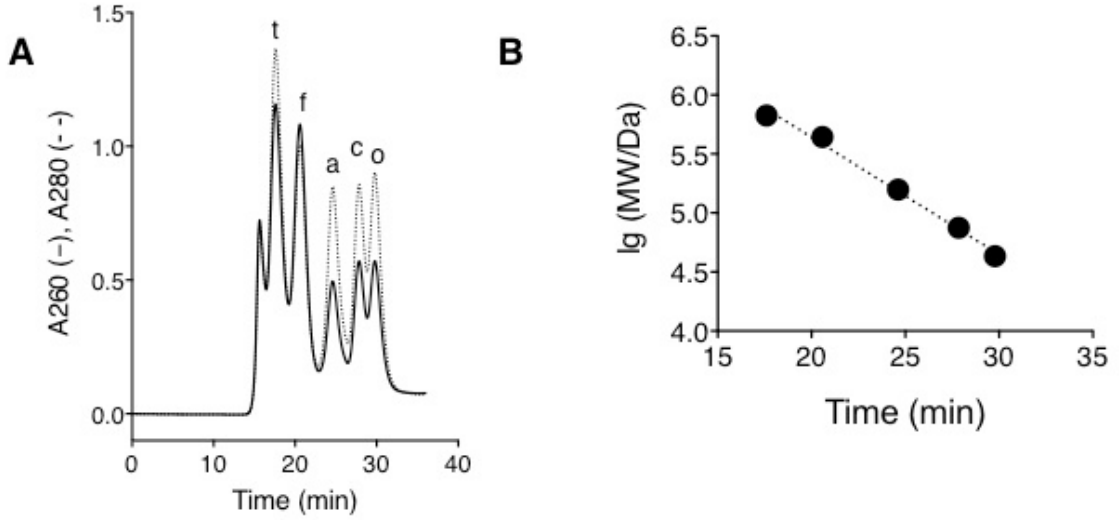


Figure S4. Gel filtration standards. (A) Elution profile of molecular weight standards: **t**hyroglobulin (669 kDa, 17.6 min); **f**erritin (440 kDa, 20.6 min); **a**ldolase (158 kDa, 24.6 min); **c**onalbumin (75 kDa, 27.8 min); **o**valbumin (44 kDa, 29.8 min). (B) Standard curve. Also see Table 1.