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Forward and Reverse Electron Transfer with the Y_{356}DOPA-β2 Heterodimer of *E. coli* Ribonucleotide Reductase

Mohammad R. Seyedsayamdost[†] and JoAnne Stubbe*[,†,‡]

Departments of Chemistry and Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139-4307

Received November 29, 2006; E-mail: stubbe@mit.edu

Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides in all organisms.[1,2] The Class I RNRs contain two subunits, α2 and β2. Each subunit is a homodimer and is required for activity. α2 contains the binding sites for NDP substrates and dNTP/ATP allosteric effectors which govern substrate specificity and turnover rate. β2 houses the diferric-tyrosyl radical (Y_{122}•) cofactor essential for radical propagation over 35 Å between the two subunits.[3] This reversible long-range hole propagation is thought to involve a pathway of conserved amino acids and perhaps the diiron cluster: [$Y_{122} \leftrightarrows$ Fe cluster $\leftrightarrows W_{48} \leftrightarrows Y_{356}$] within β2 to [$Y_{731} \leftrightarrows Y_{730} \leftrightarrows C_{439} \leftrightarrows$ nucleotide] within α2. We recently reported a method to make β2 semisynthetically using intein technology.[4] This methodology has allowed us to study radical propagation by site-specific incorporation of unnatural amino acids.[5] In one such construct, we replaced Y_{356} with 3,4-dihydroxyphenylalanine (DOPA) to generate DOPA-β2.[6] While oxidation of DOPA to a DOPA radical (DOPA•) by Y_{122}• is thermodynamically favorable by 260 mV (K_{eq} = [Y-DOPA•]/[Y•-DOPA] ≈ 2.5×10^4 at 25 °C),[7] it occurs only when the second subunit, α2, substrate and/or effector are present.[6] During the semisynthesis of DOPA-β2, a heterodimer of DOPA-β2 (DOPA-$\beta\beta'$) was produced in which the β-monomer is full length (contains residues 1−375 with DOPA at residue 356) and the β'-monomer is truncated (contains residues 1−353 only, see Supporting Information Figure S1). We now report that incubation of DOPA-$\beta\beta'$ with α2, CDP, and ATP also results in formation of DOPA• concomitant with loss of Y_{122}•. However, in contrast to full length DOPA•-β2, DOPA•-$\beta\beta'$ can reoxidize Y_{122} to Y_{122}•, demonstrating for the first time reversible electron transfer (ET) in the radical propagation chain.

The C-terminal 20 residues of β have been shown to be largely responsible for binding of β2 to α2.[8a] Previous studies by Climent et al. showed that a truncated heterodimer, in which the β'-monomer was missing the last 30 amino acids, was incapable of making dCDP in the presence of α2, CDP and ATP.[8b] In our intein wt heterodimer construct ($\beta\beta'$), in which the β'-monomer lacks the last 22 residues, we measured a specific activity ~25% that of the corresponding intein wt homodimer (β2). We therefore decided to investigate the steady state and pre-steady-state reactions of DOPA-$\beta\beta'$ with α2, substrate and effector. Design of these experiments required a knowledge of the K_d for the interaction between α2 and DOPA-$\beta\beta'$ which we determined by the method of Climent et al. to be 11 μM (see Figure S2).[8]

Activity assays of DOPA-$\beta\beta'$ revealed no dCDP formation, similar to results with the DOPA-β2 construct.[6,9] To examine the ability of DOPA-$\beta\beta'$ to generate a DOPA•, stopped flow (SF) UV−vis experiments were carried out in which DOPA-$\beta\beta'$ and CDP in one syringe were rapidly mixed with α2 and ATP in a second syringe. The reaction was monitored at 305 nm (the reported λ_{max} of a DOPA• with ϵ = 12000 $M^{-1}cm^{-1}$), and at 410 nm (the λ_{max} for Y_{122}• with ϵ = 3700 $M^{-1}cm^{-1}$).[6,10] As shown in Figure 1, the Y_{122}• (red) disappears and DOPA• (blue) grows in with similar kinetics. The data can be fit to a single exponential with a k_{obs} of 1 ± 0.2 s^{-1}, which is similar to the rate constant for the slow conformational change that gates radical propagation.[4,11] Point-by-point reconstitution of the UV−vis spectrum of the new species revealed a λ_{max} at 315 nm (see Figure S3) similar to the DOPA• observed with DOPA-β2. To confirm that the species is in fact DOPA•-$\beta\beta'$, an EPR spectrum was recorded subsequent to hand-quenching the reaction in liquid N_2 at 5 s (see Figure S4). The spectrum is identical to the one reported for DOPA•-β2.[6] Thus, DOPA-$\beta\beta'$ can generate a DOPA• in a kinetically competent fashion and requires the presence of α2, substrate, and effector.[12]

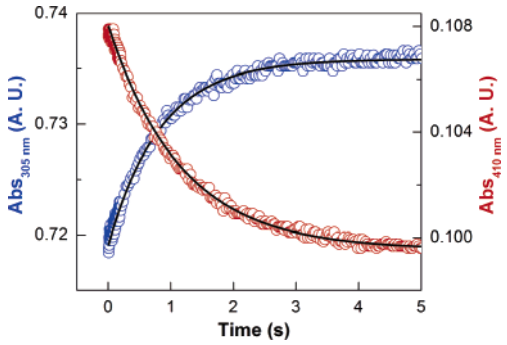

Figure 1. Kinetics of DOPA• formation (blue) and Y_{122}• disappearance (red). Black lines indicate monoexponential fits to the data.[10]

A number of differences were found between the results with DOPA-$\beta\beta'$ and those with DOPA-β2.[6] First, DOPA• formation with DOPA-β2 was multiphasic with k_{obs} of 38, 6.8, and 0.7 s^{-1}, in contrast to a single exponential at 1 ± 0.2 s^{-1} for DOPA-$\beta\beta'$. Second, in DOPA-$\beta\beta'$, the yield of DOPA• by UV−vis and EPR was 24 ± 1% and 26 ± 2% of total Y_{122}•, respectively, whereas with the homodimer, 47 and 49% of total Y_{122}• formed a DOPA•. The difference in kinetics between the two constructs suggests that deletion of the C-terminal tail in the β'-monomer reduces conformational flexibility in the α2/DOPA-$\beta\beta'$ complex, but can preserve the essential conformational change gating ET.[11] The difference in the extent of DOPA• formation may be indicative of an asymmetric interaction between the two subunits.[13,14]

We next examined the fate of the DOPA•. To increase the sensitivity of our experiment to small changes in [Y_{122}•] and [DOPA•], we developed a new method to increase the amount of diferric-Y_{122}• in our constructs from ~0.3 to 1.0−1.2 Y_{122}•s per DOPA-β2 or DOPA-$\beta\beta'$. These levels are similar to those observed in recombinant β2 (see Figure S6). We then studied the stability of the DOPA•-$\beta\beta'$ generated from incubation of DOPA-$\beta\beta'$/α2 (each 20 μM) with CDP (1 mM) and ATP (3 mM) as a function of time (Figure 2 and Table 1). The samples were hand quenched in liquid N_2 at 0.5, 2, 5, and 10 min, and the total amount of radical as well as the distribution between Y_{122}• and DOPA• was

[†] Department of Chemistry.
[‡] Department of Biology.

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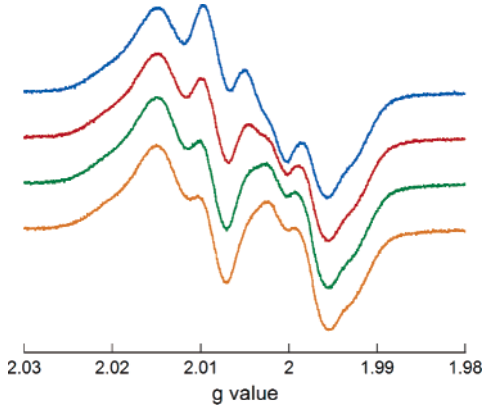

Figure 2. Fate of the DOPA• in the DOPA-$\beta\beta'$/α2/CDP/ATP complex. The reaction was initiated by mixing DOPA-$\beta\beta'$/CDP with α2/ATP and quenched by hand-freezing in liquid N_2. EPR spectra were recorded after 0.5 min (blue), 2 min (red), 5 min (green), and 10 min (orange) of reaction time. Results of the analysis of each spectrum are listed in Table 1.

Table 1. Determination of the Changes in [DOPA•] and [Y_{122}•] in the Reaction of DOPA-$\beta\beta'$/CDP with α2/ATP by EPR Methods

time[a] (min)	[spin][b] (μM)	%[c] Y_{122}•	%[c] DOPA•	[Y122•] (μM)	[DOPA•] (μM)
0	22.3	100		22.3	
0.08	22.0	72	28	15.8	6.2
0.5	21.8	73	27	15.9	5.9
2	19.8	83	17	16.4	3.4
5	19.6	87	12	17.1	2.3
10	18.8	>97	<3	>18.2	<0.5

[a] Reaction time. [b] Radical content was 1.1 per DOPA-$\beta\beta'$. EPR spin quantitation was performed using Cu^{II} as standard. [c] Spectral point-by-point subtractions were performed using Y_{122}•-DOPA-$\beta\beta'$ as reference. The % of Y_{122}• and DOPA• out of total spin at each time point is shown.

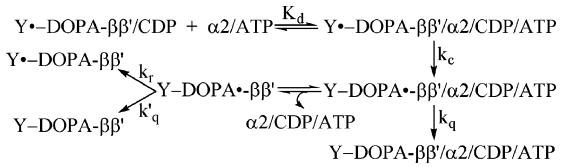

Figure 3. A model for loss of DOPA•. Y•-DOPA-$\beta\beta'$/α2/CDP/ATP exists in rapid equilibrium with Y•-DOPA-$\beta\beta'$ and α2. Addition of CDP/ATP triggers a conformational change (k_c) generating DOPA• within the complex. DOPA• can be quenched within the complex (k_q) or subsequent to dissociation of DOPA-$\beta\beta'$ from α2/CDP/ATP (k'_q). Quenching of the DOPA• also occurs by re-forming Y_{122}• via reverse ET (k_r).

determined. At 0.5 min, 27% of the total radical was present as DOPA•, and 73% as Y_{122}•. By 10 min, <3% DOPA• remained, but the amount of Y_{122}• unexpectedly increased by >14% relative to the 0.5 min signal. The experiment has been replicated five times to determine the error associated with our measurements. From these studies, we conclude that 36 ± 4% of the initially formed DOPA• is capable of regenerating Y_{122}• (k_r, Figure 3). The remaining 64% is quenched by alternative mechanisms (k_q and k'_q, Figure 3).

To examine the generality of this observation, experiments were performed on DOPA-$\beta\beta'$ with GDP/TTP, UDP/ATP, and CDP without effector, as well as on DOPA-β2 with CDP/ATP and CDP alone (see Tables S1−S6). While in all experiments DOPA• is observed, no increase in [Y_{122}•] occurred on quenching of DOPA•. Thus the conformation of α2/DOPA•-$\beta\beta'$ in the presence of CDP/ATP is unique relative to all other combinations of protein, substrate, and effector tested.

Our model to account for these observations is shown in Figure 3. It suggests that regeneration of Y_{122}• occurs upon dissociation of DOPA•-$\beta\beta'$ from α2/CDP/ATP. A unique conformation within the population of DOPA•-$\beta\beta'$s leads to reverse ET (k_r, Figure 3) and re-formation of Y_{122}• concomitant with loss of DOPA•. The ~30-fold weaker interaction between α2/DOPA-$\beta\beta'$ relative to α2/DOPA-β2 is consistent with subunit dissociation prior to back ET and with the failure to detect reverse ET with DOPA-β2.[15]

The results with DOPA-$\beta\beta'$ are of interest for several reasons. First, the amounts of DOPA• suggest that the interaction of the α2/β2 complex is asymmetric, in line with our recent observations.[6,13,14] Second, they indicate that carefully orchestrated conformational changes shift the equilibrium initially favoring Y_{122}• in DOPA-$\beta\beta'$, toward DOPA• in the complex with α2, substrate and effector. Finally, these results constitute the first direct observation of reverse ET between residues 356 and 122 in the β subunit of RNR.

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Supporting Information Available: Purification of DOPA-$\beta\beta'$, K_d determination of DOPA-β2 and DOPA-$\beta\beta'$ for α2, reconstructed UV−vis and EPR spectrum of DOPA•-$\beta\beta'$, reaction of DOPA-$\beta\beta'$ with α2 in the absence of substrate and effector monitored by SF UV−vis and EPR spectroscopies, UV−vis spectrum of DOPA-$\beta\beta'$ with 1.1 Y_{122}•/heterodimer, reaction of DOPA-$\beta\beta'$ with α2 and substrate/effector at various subunit concentrations, and analysis of reactions of DOPA-$\beta\beta'$ and DOPA-β2 with α2 and various substrate/effector pairs. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) (a) Climent, I.; Sjöberg, B. M.; Huang, C. Y. *Biochemistry* **1991**, *30*, 5164. (b) Climent, I.; Sjöberg, B. M.; Huang, C. Y. *Biochemistry* **1992**, *31*, 4801. In this study, a K_d of 13 μM was determined for the interaction of α2 with $\beta\beta'$, where the β' monomer was missing the last 30 residues.
(9) The activity assays were carried out as described in ref 5a. The specific activity of [^{14}C]-CDP was 8150 cpm/nmol.
(10) DOPA-$\beta\beta'$ (55 or 16 μM) and CDP (2 mM) were rapidly mixed in a 1:1 ratio with prereduced α2 (55 or 16 μM) and ATP (6 mM). The radical content of DOPA-$\beta\beta'$ in these experiments was 0.33 as determined by the drop-line method. Because the λ_{max} for DOPA•-$\beta\beta'$ is shifted to 315 nm, the ϵ at 305 nm was calculated as follows: $\epsilon(305) = \epsilon(315) \times (Abs_{305}/Abs_{315})$. The k_{obs} in both experiments was ~1 s^{-1}, which can be accommodated by the mechanism proposed in Figure 3.
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(12) The Y_{122}• is stable throughout the purification of DOPA-$\beta\beta'$ (~12 h). No DOPA• is observed in DOPA-$\beta\beta'$ alone by UV-vis or EPR. Further, when DOPA-$\beta\beta'$ is mixed with α2, in the absence of substrate and effector, no Y_{122}• decay or DOPA• formation is observed by SF or EPR spectroscopies (see Figure S5).
(13) Our previous studies with N_3NDP (ref 14) as well as with DOPA-β2 (ref 6) have suggested an asymmetry in the α2/β2 interaction. With DOPA-$\beta\beta'$, additional asymmetry is provided by removal of the last 22 residues in the β'-monomer. A model to account for differences in the yield of DOPA• between DOPA-β2 and DOPA-$\beta\beta'$ will be described in detail elsewhere (manuscript in preparation).
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(15) This model also provides an explanation for how 25% DOPA• can be generated with similar k_{obs}s regardless of the amount of α2/DOPA-$\beta\beta'$ complex initially formed (see Figure S7). Generation of DOPA• shifts the equilibrium to the right driving the reaction to completion. Because of fast equilibration (K_d), the rate of DOPA• formation is limited by k_c.

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