Generation of a stable, aminotyrosyl radical-induced 22 complex of Escherichia coli class Ia ribonucleotide reductase

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Generation of a stable, aminotyrosyl radical-induced α2β2 complex of Escherichia coli class Ia ribonucleotide reductase

Ellen C. Minnihan, Nozomi Ando, Edward J. Brignole, Lisa Olshansky, Johnathan Chittulu, Francisco J. Asturias, Catherine L. Drennan, Daniel G. Nocera, and JoAnne Stubbe

Ribonucleotide reductase (RNR) catalyzes the conversion of nucleoside diphosphates to deoxynucleoside diphosphates (dNDPs). The Escherichia coli class I RNR uses a mechanism of radical propagation by which a cysteine in the active site of the RNR large (α2) subunit is transiently oxidized by a stable tyrosyl radical (Y) in the RNR small (β2) subunit over a 35-Å pathway of redox-active amino acids: Y322 → [Wat7] → Y356 in β2 to Y271 → Y230 → C349 in α2. When 3-aminotyrosine (NH2Y) is incorporated in place of Y230 of a long-lived NH2Y356α2 is generated in α2 in the presence of wild-type (wt)-β2, substrate, and effector. This radical intermediate is chemically and kinetically competent to generate dNDPs. Herein, evidence is presented that NH2Y356α2 induces formation of a kinetically stable α2β2 complex. Under conditions that generate NH2Y271α2, binding between Y322NH2Y-α2 and wt-β2 is 25-fold tighter (Kd = 7 nM) than for wt-α2wt-β2 and is cooperative. Stopped-flow fluorescence experiments establish that the dissociation rate constant for the Y271NH2Y-α2wt-β2 interaction is 10−10-fold slower than for the wt subunits (~60 s−1). EM and small-angle X-ray scattering studies indicate that the stabilized species is a compact globular α2β2, consistent with the structure predicted by Uhlin and Eklund’s docking model (16). Additional support for the docking model was provided by recent structural and biochemical characterization of the active RNR complex “trapped” during turnover, and suggest that stabilization of the α2β2 state may be a regulatory mechanism for protecting the catalytic radical and ensuring the fidelity of its reactivity.

R

conformational equilibria | radical transfer | unnatural amino acid

Their model predicted a 35-Å distance between the derrick-Y122• cofactor in β2 and the active site cysteine (C349) in α2, the transient oxidation of which is a prerequisite for nucleotide reduction (1). A radical transfer (RT) pathway of conserved aromatic amino acids was proposed to account for kinetically competent radical propagation over this long distance (7). The thermodynamics of Y oxidation require loss of a proton to accompany loss of an electron, and the more detailed mechanism for proton-coupled electron transfer shown in Fig. L4 has emerged from experiments conducted in our laboratories (12, 13).

Evidence for the utilization of an amino acid pathway in long-range RT has been derived from several types of experiments. Initial site-directed mutagenesis studies of the conserved residues (Fig. L4) supported their importance in nucleotide reduction but provided little insight into the mechanism of RT (14, 15). RNR’s fidelity to a specific redox pathway involving Y356α2, Y230β2, and Y731α2 (Fig. L4) has become apparent from recent experiments in which these residues have been site-specifically replaced with unnatural amino acid analogs with modified redox properties (9, 10). For example, incorporation of 3-aminotyrosine (NH2Y) in place of the three transiently oxidized Ys in the RT pathway has been mechanistically informative. NH2Y has a reduction potential ~190 mV lower than Y at pH 7, and when incorporated at position 356 of β2, position 731 of α2, or position 730 of α2, it generates a chemically and kinetically competent NH2Y356β2 intermediate in the presence of both protein subunits, substrate, and effector. Furthermore, NH2Y RNRs are capable of making dNDPs with 3–12% activity of wild-type (wt) RNR (9, 10).

The ability to incorporate NH2Y in place of the three pathway Ys and 3-hydroxytyrosine (DOPA) in place of Y230α2 provided an opportunity to test the validity of the docking model experimentally using pulsed electron-electron double resonance (PELDOR) spectroscopy. This method allows for measurement of the distance between two weakly coupled paramagnetic species, and is applicable to RNR because of its half-site reactivity (i.e., one dNDP must be generated on the first αβ pair before RT is initiated on the second αβ pair). The PELDOR measurements of three diagonal distances between a NH2Y2• (or DOPA•) generated under turnover conditions on the first αβ pair and the Y122• remaining on the second αβ pair provided experimental support for the α2β2 docking model (16).

Additional support for the docking model was provided by recent physical biochemical studies of E. coli RNR that demonstrated...
a solution equilibrium across the three interconverting subunit states: α2 + β2 ⇌ αβ2 ⇌ αβ4 (11, 17) (Fig. 1B). The relative populations of these states were shown to depend on protein concentration and the identity and concentration of nucleotide effectors. A shape reconstruction of the small-angle X-ray scattering (SAXS) curves obtained at low micromolar protein concentrations in the presence of CDP indicated that the major species was an αβ2 complex resembling Uhlin and Eklund’s model (11). On addition of the negative activity effector dATP, or on increasing the protein concentration, the equilibrium was shifted toward an inactive αβ4 ring, as characterized by SAXS, EM, and X-ray crystallography (11, 18).

We present herein evidence that generation of NH2Y• in the reactions of NH2Y RNRs (Y730NH2Y-α2, Y731NH2Y-α2, or Y356NH2Y-β2) with the second wt subunit, substrate, and allosteric effector has been extensively studied (9, 10). One of the more striking observations is the apparent stability of NH2Y• formed at positions 730, 731, and 356, which persists on the minute time scale. The lifetimes of these on-pathway radicals, compared with the microsecond lifetimes of Y• analogs in solution (19), prompted us to investigate the cause(s) of their increased stability. X-ray crystallographic structures of Y730NH2Y-α2 and Y731NH2Y-α2 (10) indicated that the mutated pathway residues are superimposable with Y730 and Y731 in the wt structure (7), and are partially solvent-exposed in α2 alone. Similarly, Y356 is located in the C-terminal tail of β and is conformationally disordered and solvent-exposed in the structure of β2 alone (6). Thus, the long lifetimes of NH2Y• led us to surmise that the radical must be shielded from solvent, a condition that would require enhanced interaction between the subunits.

As a first test of this contention, we measured the equilibrium dissociation constant (Kd) between Y730NH2Y-α2 and wt-β2 in the presence of CDP and ATP (i.e., turnover conditions); the t1/2 of NH2Y-α2 formed under these conditions is ~2.7 min at 25 °C (20). The Kd for Y730NH2Y-α2 was determined using a spectrophotometric competitive inhibition assay (8) in which the wt-α2wt-β2 interaction is disrupted by titrating increasing concentrations of an inhibitor (Y730NH2Y-α2) into the assay mixture. The corresponding decrease in RNR activity is representative of the amount of inhibitor in the complex, and is used to extrapolate a Kd for the Y730NH2Y-α2wt-β2 interaction. A plot of [Y356NH2Y-α2]bound vs. [Y356NH2Y-α2]free is sigmoidal, rather than the hyperbolic curve observed for the wt-α2wt-β2 interaction. Analysis of the data by the Hill model for cooperative binding gives Kd = 7 nM for the Y356NH2Y-α2wt-β2 interaction (Fig. 2, red). This Kd is 26-fold tighter than the wt-α2wt-β2 (0.18 μM) interaction measured by the same method using the inactive mutant Y122-Fβ2 as a competitive inhibitor of the wt association (14). A Kd = 8 nM was determined for association between the analogous affinity-tagged protein, His6-Y730NH2Y-α2 (10), and wt-β2, indicating that the N-terminal His6-tag does not disrupt the subunit interactions. The Kd between Y731NH2Y-α2 and wt-β2 and those between Y356NH2Y-β2 and wt-β2 were also determined. In both cases, the binding was cooperative, with Kd = 17 nM and Kd = 0.30 μM, respectively (Fig. 2, blue and green). Thus, the Y356NH2Y-α2wt-β2 interaction is 10-fold stronger than the corresponding wt interaction. The basis for the
weaker $K_d$ between Y356NH$_2$Y-$\beta$2 and wt-$\alpha_2$ is not understood at present but may reflect decreased stability of the NH$_2$Y at this position compared with positions in $\alpha_2$.

We further hypothesized that the origin of the enhanced interaction between Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ rests in the ability to generate the stable pathway radical, NH$_2$Y$_{730}$, rather than in ground-state structural differences between Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\alpha_2$. To this end, the $K_d$ for the interaction between Y$_{731}$F/Y$_{739}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ was also measured, because the additional Y$_{731}$F mutation prevents NH$_2$Y formation (21). The data (Fig. S1) reveal a loss of cooperativity and a $K_d = 1.14$ $\mu$M, which is $\approx 160$-fold weaker than that for the Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ interaction. This result supports the model that formation of an on-pathway NH$_2$Y stabilizes the subunit interaction.

A nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography “pull-down” assay was designed to validate the enhanced interaction between Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ independently. A 1:1 mixture of His$_6$Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ (10 $\mu$M) was combined with CDP and ATP under single-turnover conditions (i.e., no reducer) to generate NH$_2$Y$_{730}$, and the reaction mixture was incubated for 30 s with Ni-NTA agarose. The resin was washed to remove nonspecifically bound proteins, and bound protein(s) were then eluted with high [imidazole]. The entire procedure was complete within 3 min of initial mixing. The interaction between Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ was examined under identical conditions. The protein content after each step was determined by SDS/PAGE (Fig. S2), and comparative densitometry analysis revealed that twice as much wt-$\beta_2$ coeluted with His$_6$Y$_{730}$NH$_2$Y-$\alpha_2$ as with His$_6$-Y$_{730}$NH$_2$Y-$\alpha_2$ (wt), consistent with a Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction that is stronger than the wt subunit interaction.

**Kinetics of Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction.** The long $t_{1/2}$ of NH$_2$Y$_{730}$-suggested that the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction is tight and kinetically stable. To investigate this possibility, the $K_d$s for various $\alpha_2$ subunit interactions were measured by SF fluorescence spectroscopy, with the prediction that the $k_{off}$ for Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ would be significantly lower than that for wt-$\alpha_2$-wt-$\beta_2$. We used DAN-$\beta_2$(V$_{565}$C), a mutant $\beta_2$ labeled with an environmentally sensitive fluorophore at a minimally disruptive position on the C-terminal tail (22), as a competitive inhibitor of $\beta_2$ to measure $k_{off}$. Four subunit interactions were measured in the presence of CDP and ATP: wt-$\beta_2$ with wt-$\alpha_2$ or Y$_{730}$NH$_2$Y-$\alpha_2$; reduced-diferic-$\beta_2$ (met-$\beta_2$, incapable of initiating RT) and Y$_{730}$NH$_2$Y-$\alpha_2$ with wt-$\beta_2$ or met-$\beta_2$. For each experiment, $\alpha_2$ (1 eq), unlabeled $\beta_2$ (3 eq), and CDP were combined in the absence of a visible signal and rapidly quenched with an excess of DAN-$\beta_2$(V$_{565}$C) (105 eq) in the second syringe, and the total fluorescence at wavelengths $>420$ nm was monitored. An increase in fluorescence intensity in this regime reports on the displacement of the fluorophore to a more hydrophobic environment, as is predicted to occur when the disordered $\beta$-tail binds to $\alpha$ (22). The averaged kinetic traces for the wt-$\beta_2$-wt-$\alpha_2$ and met-$\beta_2$-met-$\alpha_2$ reactions are shown in Fig. S4 and Fig. S3A, respectively. Monoeponential fits to the data give $k_{off} = 74$ s$^{-1}$ and $k_{off} = 63$ s$^{-1}$, respectively. The similarity between these rate constants indicates that the presence of either Y$_{730}$ or for the active site disulfide formed during turnover has a significant impact on $k_{off}$. The averaged fluorescence trace of an analogous experiment with Y$_{730}$NH$_2$Y-$\alpha_2$ and met-$\beta_2$ (Fig. S3F) gave a $k_{off} = 38$ s$^{-1}$, with a total fluorescence change similar to that of the wt-$\alpha_2$ reactions. Thus, $k_{off}$ of Y$_{730}$NH$_2$Y-$\alpha_2$ from met-$\beta_2$ is approximately twofold slower than with wt-$\alpha_2$, even in the absence of NH$_2$Y$_{730}$ formation.

Finally, $k_{off}$ was measured for Y$_{730}$NH$_2$Y-$\alpha_2$ and wt-$\beta_2$ in the presence of CDP and ATP, conditions that produce the long-lived radical. In contrast to the previous experiments, there was no significant increase in fluorescence over the first 150 ms (Fig. 3B, Inset), and the small amplitude change observed over longer times (Fig. 3B, 2 s) constitutes $<15\%$ of the total fluorescence change observed over 150 ms in the three previous experiments. This change may reflect interaction of DAN-$\beta_2$(V$_{565}$C) with the small fraction (<20%) of unbound Y$_{730}$NH$_2$Y-$\alpha_2$ in the initial solution (10). Details regarding the quantitative assessment of the amount of Y$_{730}$NH$_2$Y-$\alpha_2$ interacting with wt-$\beta_2$ in our experiments are provided in SI Materials and Methods. Because the $k_{off}$ for the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction was too slow to measure on the SF time scale, steady-state fluorescence spectroscopy was conducted under identical conditions. A slow fluorescence increase was observed over 40 min; no increase was observed on this time scale for the wt-$\alpha_2$-wt-$\beta_2$ control (Fig. S4). Fitting the data gives a $k_{off}$ on the order of $10^{-5}$ s$^{-1}$, which is in agreement with the decay constant for NH$_2$Y$_{730}$-($4 \times 10^{-5}$ s$^{-1}$) and suggests that the lifetime of the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction and the pathway radical are correlated. The combined fluorescence experiments establish that the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction is kinetically stable compared with the wt subunit interaction.

**Structure of Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ Complex Characterized by EM.** The strength and kinetic stability of the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction suggested that the complex might be visualized by EM, potentially providing valuable information about the structure and oligomeric state of the association. Y$_{730}$NH$_2$Y-$\alpha_2$ was combined with wt-$\beta_2$ in the presence of CDP and ATP, applied to the EM grid with minimal incubation time relative to the lifetime of the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction and the pathway radical are correlated. The combined fluorescence experiments establish that the Y$_{730}$NH$_2$Y-$\alpha_2$-wt-$\beta_2$ interaction is kinetically stable compared with the wt subunit interaction.

The resulting class averages were compared with projections of an $\alpha_2$ crystal structure (23) or the $\alpha_2$ docking model (7) (Fig. 4 A and B and Fig. S5). This comparison suggests that the majority of the averages represent different views of a complex that adopts a subunit arrangement consistent with the $\alpha_2$ docking model. Several other averages clearly represent views of the free $\alpha_2$ subunit. To determine whether observation of the $\alpha_2$ complexes is dependent on NH$_2$Y$_{730}$ formation, we prepared a specimen with met-$\beta_2$ substituted for wt-$\beta_2$. Strikingly, the averages from this control experiment almost exclusively correspond with dissociated $\alpha_2$ and $\beta_2$ subunits (Fig. 4C), indicating that NH$_2$Y$_{730}$ formation is a prerequisite for visualization of the $\alpha_2$ complex. Some $\alpha_2$ class averages correspond to orientations in which subunits are not overlapping and can be easily identified. These views offer an opportunity to understand the subunit arrangement in the $\alpha_2$ complex better, especially when considered relative to a 3D map calculated from tilted-pair particle images (24). X-ray models of $\alpha_2$ and $\beta_2$ were separately fit into the 3D EM map (Fig. 4D) and compared with fitting of the $\alpha_2$ docking model (7) as a single rigid unit (Fig. 4E). The results from these two approaches agree [within the limitations related to low (~32 $\AA$) resolution and possible stain-induced deformation of the EM map], providing direct experimental support for the docking model.
Free subunits were first characterized under identical conditions by Guiner and Kratky analyses, as described previously (11). Although a small amount of inherent aggregation was detected in Y<sub>730</sub>NH<sub>2</sub>-Y-α2, q ranges with well-defined radii of gyration (R<sub>q</sub>) could be identified in all samples by automated Guiner analysis (25) (Fig. S6 A–D). This analysis yielded similar extrapolated R<sub>q</sub> values for both forms of β (30.2 ± 3.7 Å for wt and 31.1 ± 1.4 Å for met), as well as for the two forms of α (42.0 ± 4.3 Å for wt and 45.2 ± 5.4 Å for Y<sub>730</sub>NH<sub>2</sub>Y). Shape information could be readily acquired from Kratky analysis of the mid-q region of scattering data, which is unaffected by the presence of aggregates (Fig. S7). Scattering from folded, globular species decays as q<sup>−2</sup>, which gives rise to a pronounced peak when plotted in Kratky representation (h<sup>2</sup> vs. q), whereas multimodal peaks are indicative of distinct, folded domains in a nonglobular arrangement (26). In the case of wt-β2 and met-β2, monomodal peaks are observed, consistent with the compact monomer arrangement within the β2 dimer. Furthermore, the curves are nearly superimposable, indicating that the radical does not affect the shape of this subunit. By comparison, the Kratky curves of wt-α2 and Y<sub>730</sub>NH<sub>2</sub>-Y-α2 display peaks with shoulders, consistent with the less globular monomer arrangement in α2 dimers. Importantly, the curves are nearly identical in shape, indicating that the mutation does not change the overall shape or fold of α2.

Interactions between subunits were examined under conditions in which mixtures of α2β2 and α4β4 are expected to form based on previous work with wt-α2 and met-β2 (11), namely, at a high protein concentration (30 µM) in the presence of GDP and dTTP. Guiner and Kratky analyses were again used. Previously, it was shown that the globular α2β2 complex (R<sub>q</sub> ~40 Å) has a monomodal Kratky curve with a peak at q ~0.04 Å<sup>−1</sup>, whereas the highly nonglobular αβ<sub>2</sub> complex (R<sub>q</sub> ~70 Å) has a multimodal Kratky curve with distinct peaks at q ~0.024 Å<sup>−1</sup> and ~0.06 Å<sup>−1</sup> (11). Mixtures of α2β2 and α4β4 thus lead to apparent R<sub>q</sub> between 40 and 71 Å and a smearing of Kratky peaks. Qualitatively, greater definition of the curves indicates higher levels of α4β4, whereas sharpening of the Kratky curve to a single peak at q ~0.04 Å<sup>−1</sup> indicates higher levels of α2β2.

A reaction mixture representing a preturnover state consisting of 30 µM wt-α2 and met-β2 with dTTP/GDP led to a bimodal Kratky curve and a corresponding R<sub>q</sub> of 61.6 ± 0.1 Å, suggestive of a α2β2/α4β4 mixture (Fig. 5, red) and consistent with previous observations (11). Replacing met-β2 with wt-β2 resulted in a similar Kratky curve (Fig. 5, blue) with minimal changes to the R<sub>q</sub> (60.3 ± 0.2 Å) and q<sup>2</sup> thus lead to apparent R<sub>q</sub> between 40 and 71 Å and a smearing of Kratky peaks. Qualitatively, greater definition of the curves indicates higher levels of α4β4, whereas sharpening of the Kratky curve to a single peak at q ~0.04 Å<sup>−1</sup> indicates higher levels of α2β2.

Recent analytical ultracentrifugation and SAXS experiments in which an α2β2 complex resembling the docking model is an intermediate between the subunit dissociated state and the inhibited α4β4 state (11) (Fig. 1B). In the wt model, a shift in equilibrium toward α4β4 occurs in the presence of increasing protein concentrations or inhibitory concentrations of dATP. The enhanced stability of the Y<sub>730</sub>NH<sub>2</sub>-Y-α2wt-β2 interaction should lead to increased abundance of α2β2 relative to the other states. To test this hypothesis and to examine the contribution(s) of RT to the stability of the α2β2 complex, we investigated the distribution of solution species formed by various combinations of α2 (wt and Y<sub>730</sub>NH<sub>2</sub>Y), β2 (wt and met), substrate, and effector by SAXS. Data were processed as described previously (11) to yield the scattering intensity, I, vs. q, a function of scattering angle.

Structure of Y<sub>730</sub>NH<sub>2</sub>-Y-α2wt-β2 Complex and Its Quaternary Equilibria Characterized by SAXS. Recent analytical ultracentrifugation and SAXS experiments in which an α2β2 complex resembling the docking model is an intermediate between the subunit dissociated state and the inhibited α4β4 state (11) (Fig. 1B). In the wt model, a shift in equilibrium toward α4β4 occurs in the presence of increasing protein concentrations or inhibitory concentrations of dATP. The enhanced stability of the Y<sub>730</sub>NH<sub>2</sub>-Y-α2wt-β2 interaction should lead to increased abundance of α2β2 relative to the other states. To test this hypothesis and to examine the contribution(s) of RT to the stability of the α2β2 complex, we investigated the distribution of solution species formed by various combinations of α2 (wt and Y<sub>730</sub>NH<sub>2</sub>Y), β2 (wt and met), substrate, and effector by SAXS. Data were processed as described previously (11) to yield the scattering intensity, I, vs. q, a function of scattering angle.

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**Fig. 4. Visualization of the NH<sub>2</sub>-Y<sub>730</sub>-stabilized α2β2 complex by EM.** (A) Class averages of Y<sub>730</sub>NH<sub>2</sub>-Y-α2 with wt-β2 in the presence of CDP and ATP. (B) Two-dimensional projections created from dTTP/GDP-bound α2 crystal structure [Protein DataBank (PDB) ID code 4R1R (23) (blue outline)] and the α2β2 docking model (7) (red outline) that best correlate with the class averages in A. (C) Class averages of Y<sub>730</sub>NH<sub>2</sub>-Y-α2 with met-β2 in the presence of CDP and ATP. (D) Three-dimensional reconstruction from tilted views (gray) of the particles in the class marked by the asterisk in A. Structures of α2 and β2 (PDB ID codes 4R1R and 1RIB) iteratively fit into the reconstruction are shown in ribbons (α2 chains in light and dark blue with activity sites in green, β2 chains in red and orange). (E) Three-dimensional reconstruction as in D fit with the α2β2 docking model. Averages and projections are 209 Å wide at the specimen level.
(175 μM) was then added to the reaction, and the mixture was monitored every 2 min for a total of 22 min. Initially, a largely monomodal peak was observed in the Kratky curve (Fig. 5C, blue), demonstrating the stability of this α2β2 complex even under strongly inactivating conditions. With time, the Kratky curve slowly becomes more bimodal (Fig. 5C, red), approaching the state observed with wt-α2 and wt-β2 (Fig. 5B). Singular value decomposition analysis indicates that the data can be described as a two-state transition that is not completed over the course of 22 min (Fig. 59), whereas the change in shape of the Kratky curve is consistent with the conversion of α2β2 to α4β4. These results are consistent with very slow dissociation of the α2β2 complex, even under strongly inhibitory conditions.

Catalytic Activity of the Y730NH2Y-α2wt-β2 Complex. We next investigated whether the stabilized complex is an active form of the enzyme. A modified RNR activity assay was conducted in which the Y730NH2Y-α2wt-β2 complex was preformed by addition of His6-Y730NH2Y-α2 to a mixture of wt-β2 (2 eq), CDP, and ATP (mix 1). This solution was briefly aged and then diluted into a second solution containing the components of the standard steady-state RNR assay, including additional β2 (5 eq) and the reducing agents required for multiple turnovers, thioredoxin (TR), thioredoxin reductase (TRR), and NADPH (mix 2) (9). The activity of His6-Y730NH2Y-α2 from the preformed NH2-Y-α2 wt-β2 complex was 86 ± 10 mmol·min⁻¹·mg⁻¹. As a positive control, wt-β2 was omitted from mix 1 (i.e., no preformed complex) but included in mix 2. Under these conditions, His6-Y730NH2Y-α2 had a specific activity of 88 ± 1 mmol·min⁻¹·mg⁻¹, consistent with previous reports (10). This result indicates that the Y730NH2Y-α2 wt-β2 complex induced by NH2-Y-α2• formation is either active itself or exists in rapid equilibrium with an active form of the complex. The fluorescence experiments indicate that koff for the complex is slow compared with the steady-state koff. This observation suggests it is the Y730NH2Y-α2wt-β2 complex itself that is an active quaternary structure. However, the effect of reductant(s) on the stability of the Y730NH2Y-α2wt-β2 complex has not been studied and could influence koff in the steady state.

Discussion

The results described herein provide very strong evidence that the reaction of Y730NH2Y-α2, wt-β2, substrate, and effector generates a kinetically stable α2β2 complex and that the stability of this complex originates from the formation of a radical, NH3-Y-α2• on the RT pathway. Remarkably, the transfer of the equivalent of a single hydrogen atom from NH3-Y730 in α2 to Y122• in β2 in active RNR increases the kinetic stability of a 260-kDa complex by a factor of ~10^7 relative to wt-α2. Recent studies have indicated that the affinity between E. coli α2 and β2 is increased in the presence of matched substrate/effector pairs and that the binding of substrate/effector to α2 conformationally gates radical initiation in β2, indicating a role for nucleotide-induced conformational triggering of RT in the active α2β2 complex (10, 27). The present studies extend this hypothesis by demonstrating that RT, in turn, transiently induces tight association between the subunits. We rationalize that this enhanced subunit affinity evolved as a regulatory mechanism to prevent quenching of transiently formed Y•s, which would result in RNR inactivation through loss of the catalytic oxidant.

Although the current studies have focused specifically on Y730NH2Y-α2, studies with other RNRs containing unnatural Y analogs suggest that localizing a radical on the pathway is a general mechanism for strengthening and stabilizing α2β2 interactions. For example, we have recently reported the replacement of Y122• with several high-energy radical initiators (NO2Y122• or F2Y122•). Incubation of these mutant β2s with wt-α2, substrate, and effector converts up to 50% of the initial radical at position 122 to a Yα• intermediate (28, 29). The lifetime of the Y356• depends on the identity of the radical initiator at position 122; however, in all cases, the new radical persists on the minute time scale. Recalling that Y356•, located on the C-terminal tail of free β2, would likely be reduced on the microsecond time scale (19), the observation of a long-lived Y356• suggests a comparably long-lived α2β2 association. In retrospect, a similar stabilization of the α2β2 complex likely accounts for the observations with the mechanism-based inhibitor 2'-azido-2'-deoxynucleotide, which reacts in the presence of α2 and β2 to generate a nitrogen-centered, nitrogen-centered radical (N•) in the enzyme active site. Fifty percent of the total initial Y• is rapidly lost concomitant with 50% formation of the N• and 90% loss of total enzyme activity, consistent with the half-site reactivity model. The complete loss of Y•, however, occurs only after 30 min, an observation suggestive of very slow subunit dissociation before complete Y• quenching (30).

The characterization of a stable, NH2Y•-induced complex has provided a unique opportunity to visualize the active α2β2 complex “trapped” during catalysis by EM. Although the experiment was challenging due to the kinetic complexity and extent of NH2Y• formation, class averages and 3D reconstructions reveal a compact, globular structure consistent with the docking model. SAXS experiments provide additional support for the solution stabilization of a Y730NH2Y-α2wt-β2 complex. These experiments, in combination with the previous PELDOR distances (16) and SAXS data (11), establish the docking model as an accurate representation of the active RNR structure.

In summary, the results presented above provide a visual representation of the active structure of the E. coli class Ia RNR. They also indicate that subtle conformational changes, which are induced by nucleotide binding and long-range RT, regulate RNR by preventing quenching of the catalytic radical at positions along the pathway, a loss that would result in enzyme inactivation. This mechanism is one of many that E. coli RNR uses to ensure the fidelity of its complicated radical chemistry.
5F Fluorescence Experiments. DAN-[2V(35S)C] was prepared according to the protocol reported in the literature (22). 5F fluorescence experiments were performed at 25 °C as described previously (32). In all cases, one syringe contained u2 (wt or Y53H, Y60, 0.16 μM), u2 (wt or met, 0.5 μM), CDP (1 mM), and ATP (3 mM) in assay buffer. A second syringe contained DAN-[2V(35S)C] (17.5 μM) in assay buffer. The contents of the syringes were mixed 1:1, and the fluorescence was monitored at >420 nm.

Structural Characterization by EM. Y53H,Y60,Y72 (1.5 μM), wt-u2 or met-u2 (3 μM), ATP (3 mM), and CDP (1 mM) were combined in assay buffer. The reaction mixture (8 μL) was applied to a carbon-coated EM grid, rinsed with assay buffer/ATP/CDP, and stained with a 2% uranyl acetate solution that also contained 0.2% tannic acid. The specimens were imaged at 120 kV on a Tecnai F20 electron microscope (FEI) equipped with an UltraScan 4000 CCD camera (Gatan) using Legoion (33) operated in manual low-dose mode at a magnification of 50,000× with a pixel size of 2.18 Å at the specimen level. For the preparation with wt-u2, 48 pairs of images of the untilted and −55° tilted specimen were collected, and an initial dataset of 26,845 particle pairs was windowed from the CCD frames and processed using SPIDER (34). For the preparation with met-u2, 2,187 particles were windowed from 5 untilted images using SPIDER, and they were aligned and classified using the iterative Stable Alignment and Clustering program in SPARX (35).

SAXS Analysis. SAXS data were collected using a Pilatus 100-K detector and an in vacuo 2-mm pathlength quartz capillary flow cell (36) at the Cornell High Energy Synchrotron Source (CHESS) G1 station. Data were processed following previously described protocols (11, 37). The momentum transfer variable 4πsinθ/λ was defined as q = 4πsinθ/λ, where λ is the X-ray wavelength and 2θ is the scattering angle with respect to the beam. Reaction components were mixed immediately before data acquisition. For each sample, exposures that did not exhibit time-dependent changes were averaged. All samples were in assay buffer with the nucleotide concentrations specified in the figure legends.

Activity Assays. His6-Y53H,Y60,Y72 (5 μM), wt-u2 (10 μM), [3H]-CDP (1 mM, 20,000 cpm/mmol), and ATP (3 mM) in a total volume of 25 μL were mixed in assay buffer at 25 °C, and the resulting solution was aged for 30 s. A fraction of this mixture (20 μL) was then diluted into a mixture (200 μL) containing additional wt-u2 (2.5 μM), [3H]-CDP (1 mM), ATP (3 mM), TR (0.5 μM), NAPDH (1 mM) in assay buffer, and RNR activity was measured (9).

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