Nucleotide-dependent Formation of Catalytically Competent Dimers from Engineered Monomeric Ribonucleotide Reductase Protein R1*

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Each catalytic turnover by aerobic ribonucleotide reductase requires the assembly of the two proteins, R1 (α2) and R2 (β2), to produce deoxyribonucleotides for DNA synthesis. The R2 protein forms a tight dimer, whereas the strength of the R1 dimer differs between organisms, being monomeric in mouse R1 and dimeric in Escherichia coli. We have used the known E. coli R1 structure as a framework for design of eight different mutations that affect the helices and proximal loops that comprise the dimer interaction area. Mutations in loop residues did not affect dimerization, whereas mutations in the helices had very drastic effects on the interaction resulting in monomeric proteins with very low or no activity. The monomeric N238A protein formed an interesting exception, because it unexpectedly was able to reduce ribonucleotides with a comparatively high capacity. Gel filtration studies revealed that N238A was able to dimerize when bound by both substrate and effector, a result in accordance with the monomeric R1 protein from mouse. The effects of the N238A mutation, fit well with the notion that E. coli protein R1 has a comparatively small dimer interaction surface in relation to its size, and the results illustrate the stabilization effects of substrates and effectors in the dimerization process. The identification of key residues in the dimerization process and the fact that there is little sequence identity between the interaction areas of the mammalian and the prokaryotic enzymes may be of importance in drug design, similar to the strategy used in treatment of HSV infection.

Ribonucleotide reductase (RNR)† is a multimeric enzyme that uses long range electron transfer from the metal center in the R2 protein to the active site in the R1 protein to perform the reduction of ribonucleotides to deoxyribonucleotides by help of radical chemistry. Being the only provider of these essential components for de novo synthesis of DNA, RNR is expressed ubiquitously in the cytosol of all living cells. The active enzyme is a tetramer (α2β2) formed by two structurally and functionally different homodimeric proteins, denoted R1 (α2) and R2 (β2) (1).

RNR reduces all four common ribonucleotides, thus a strict control system is required to maintain a balanced supply of deoxyribonucleotides. This control is mediated by a sophisticated allosteric regulation via two functionally different nucleotide binding sites in the R1 protein: the specificity site that dictates substrate selection by binding of effectors (dTTP, dGTP, dATP, or ATP), and the activity site that determines global enzymatic activity via effector (ATP or dATP) binding (2). In addition to the allosteric regulation, activity is also controlled via cell cycle regulation (3, 4). Differences in structure, mechanism of catalysis, oxygen dependence, and substrate phosphorylation level divide ribonucleotide reductases into three classes (1). Class I, which is the focus of this study, is found in all Eukaryota (except Euglena) and in many Eubacteria (5–7). Differences in allosteric regulation further divide the class I RNRs into two subclasses, Ia and Ib. The eukaryotic and approximately half of the sequenced eubacterial enzymes groups in class Ia. A few closely related γ-proteobacterial genomes, including that of Escherichia coli, encode both class Ia and class Ib operon. Only class Ia can support proliferation of E. coli under a variety of laboratory growth conditions (8).

The E. coli class Ia RNR is a well studied system in aspects of structure, allosteric regulation, and catalytic mechanism, and it serves as a model for class I RNRs in other organisms. The E. coli R1 monomer has a molecular mass of 85.7 kDa and consists of 761 amino acids, arranged in three domains: one mainly helical N-terminal domain (~220 residues), a 10-stranded αβ barrel domain (~480 residues), and a 70-residue βαβαβ domain, which covers the bottom of the barrel (9, 10). The active site is located in a cleft across the protein between the N-terminal and the barrel domain. The interaction area between the two R1 polypeptides is formed by the barrel helices βα (residues 234–250) and αβ (residues 279–289), which are connected to the βB strand (residues 253–257) via two loop regions (residues 251–252 and 258–278). The longer of these loop regions (called loop 1) and part of the loop region subsequent to αβ (residues 290–297, called loop 2) may also contribute to the dimer interaction as well as to the interaction of R1 with protein R2 (cf. Fig. 1). The dimer interaction is stabilized mainly by van der Waals contacts (Fig. 1B). Only a few interprotein hydrogen bonds and charged interactions can be found. Compared with the size of the R1 dimer (110 × 75 × 75 Å) these
interactions are weak, and the buried surface area of 2600 Å² (corresponding to 4% of the accessible surface of the monomers) is relatively small. Because of this it is obvious that the domains of the subunits can change their localization in relation to each other.

The *E. coli* R2 monomer of 43.4 kDa comprises 375 amino acids (11). Each monomer is made up of 13 \( \alpha \) helices and two \( \beta \) strands (Fig. 1A). Tyr-122 that harbors the radical is buried within each monomer close to a di-iron center (Fig. 1A). Compared with R1, the dimer interaction in R2 is much tighter and has 18.5% of the accessible surface of the monomers buried in the dimer (11). The interaction area has a hydrophobic patch in the center, which is surrounded by polar and charged interactions. Homology studies of different species have shown that neither the R2 protein-protein interactions nor the homology of interacting residues at the interface are conserved (11).

The interaction between R1 and R2 has been shown both biochemically (12) and in the crystal structure (9) to be dependent on the C-terminal part of protein R2. The crystal structure further revealed that the dimeric structure of R1 is complementary to a part of the R2 dimer, which led Uhlin and Eklund (9) to build a model of the \( \alpha_2\beta_2 \) holoenzyme (Fig. 1A). So far no crystal structure of the *E. coli* holoenzyme complex could be obtained; however, the holoenzyme from *Salmonella typhimurium* was recently solved at a resolution of 4 Å (12). It partly supports the Uhlin and Eklund model. RNR from *S. typhimurium* belongs to class Ib, which lacks the 50 N-terminal residues and consequently has lost the allosteric activity site (2). The Uhlin and Eklund model of the *E. coli* class Ia RNR (9) provides a structural basis for theories concerning the allosteric regulation and catalytic mechanisms of the holoenzyme. Notably, the active site cysteine residues sit in the inner parts of the substrate pocket of R1, and the rigid structure of the \( \alpha\beta \) barrel makes it impossible to move them closer to the stable tyrosyl free radical in R2 by modeling (5). These points led to the theory of a long range radical transfer pathway (7, 9, 13). Such a reaction mechanism was substantiated by the observation that nine highly conserved amino acid residues constitute this pathway and that mutation in any of the nine residues inactivates the radical transfer (14). This means that a complex of R1-R2 is necessary to form an active holoenzyme.

Sequence alignments between *E. coli* and mouse R1 proteins show an overall high homology and therefore suggest a structural similarity. Nevertheless, the monomer-dimer equilibrium in solution seems to be different between the two. Purification of R1 protein from *E. coli* showed that its predominant structure is dimeric in the absence of any ligands or effectors (15), which led to the assumption that the \( \alpha_2\beta_2 \) tetramer is the major
active form of the enzyme in *E. coli* (9). Purification of R1 protein from mammalian sources, e.g. mouse (16, 17), calf thymus (18), and Ehrlich tumor cells (19), showed that the protein appeared mostly in the monomeric form (20). Further studies showed that the oligomerization status of mouse R1 is dependent on ligand binding to the allosteric specificity site (18, 21, 22). This is concordant with structural studies on *E. coli*, placing the specificity site at the R1 dimer (α2β2) interface (23). It also indicates that oligomerization and formation of an active holoenzyme is regulated by effectors close to the interface, which is supported by the fact that this area is sensitive to structural changes. In general, binding to R2 could also stabilize a loose dimer formation between R1 monomers (20). The dissociation constant for the initial binding event between *E. coli* R1 and R2 (i.e. between one polypeptide of each homodimer) is 13 μM, whereas the *Kd* of the fully associated holoenzyme is 0.2 μM (24). The aim of this study was to investigate how the dimer interaction area of protein R1 is constructed to gain a better understanding of the assembly process of NRN in *E. coli* and to explain differences in oligomerization status of R1 proteins from different species.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—***NovaBlue E. coli* (recA, endA, lac*) from Novagen were used for site-directed mutagenesis. *E. coli* BL21DE3 (B F- deo T, omplT, hsdRr3 mB, gal (DE3)) from Novagen were used for overexpression. Site-directed mutagenesis experiments were performed on wild type pTB1 plasmid (Ch’), which is a pT718R plasmid derivative and contains the *nrda* gene (25). Plasmid pLYsS-(Cm*) DNA was purchased from Novagen.

**Growth Medium—**Both strains were grown in Luria or Terrific Broth (TB) medium. The BL21DE3/pLYsS strain used for overproduction of mutant R1 protein was grown in the presence of carbencillin (50 μg/ml) and chloramphenicol (34 μg/ml) and 2 drops of antifoam.

**Site-directed Mutagenesis on Double-stranded DNA—**The site-directed mutagenesis was performed on pTB1 plasmids using the QuikChange site-directed mutagenesis kit by Stratagene with proof-reading Pfx-DNA polymerase and standard oligonucleotide primers obtained from Invitrogen (Table 1).

The sequences of the modified *nrda* genes were verified by DNA sequencing using the DYEnamic ET terminator kit (MegaBACE) from Amersham Biosciences. Sequencing gels were run at the Department of Cellular and Molecular Biology at the Karolinska Institute, Stockholm.

No secondary mutations were found in the *nrda* gene of any of the mutated pTB1 plasmids.

**Expression of Mutant Protein—**Each modified R1 protein was expressed from its pTB1 plasmid in BL21DE3/pLYsS cells. Typically, 1-liter cultures in LB or TB medium were grown at 37 °C with shaking in 5-liter flasks for at least three doublings. At an *A600* of 0.4 protein expression was induced by addition of isopropyl-β-thiogalactopyranoside to a final concentration of 1 mM (26). Growth proceeded for 3 h, then the cells were harvested by centrifugation and stored at −80 °C. The protein concentration in 0.1 mg of cells was analyzed on SDS-PAGE using the Amersham Biosciences Phast system.

**Protein Extraction and Purification—**Frozen cells were disintegrated in a X-press and resuspended in a buffer containing 50 mM Tris, pH 7.6, 20% glycerol, 10 mM MgCl2, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride. Nucleic acids in the crude extracts were precipitated by addition of neutralized streptomyacin sulfate to a final concentration of 2%. After centrifugation the proteins of the supernatant were precipitated by addition of ammonium sulfate to a saturation of 60% and collected by centrifugation. The pellet of ammonium sulfate precipitated protein was dissolved in 60% of buffer A (0.75 mM (NH₄)₂SO₄ and 5 mM DTT) and 40% of buffer B (50 mM Tris-HCl, pH 7.6, and 5 mM DTT) and loaded onto a 20-ml bed volume HiLoad® 16/10 Phenyl-Sepharose® High Performance column to obtain a standard curve for determination of molecular masses.

**Gel Filtrations in the presence of Effectors—**Wild type R1 and N238A proteins were analyzed in the same running buffer as above supplemented with one of the following nucleotides: 100 μM dTTP, 0.1 μM dATP, 1 μM dGTP, 1.5 mM dCTP, 0.5 mM GDP, or with a combination of 100 μM dTTP and 0.5 mM GDP. Standard curves for each nucleotide(a) condition were performed as described above.

**Activity Assays—**Ribonucleotide reductase activity was measured by the spectrophotometric assay or by the [3H]CDP assay as described previously (27). Typically, assays contained 1.5 mM ATP, 11 mM Mg(Ac)₂, 0.4 mM NADPH, 13 μM thioredoxin, 0.5 μM thioredoxin reductase, 30 μM EDTA, 34 mM HEPES, pH 7.6, and 0.05 μM R2 protein. The R1 concentration was varied between 0.06 and 0.8 μM, and the enzyme reaction was started by addition of cold or tritiated CDP substrate to a final concentration of 0.5 mM. The consumption of NADPH was monitored during 5–10 min in the spectrophotometric assay, whereas the [3H]CDP assay was stopped after 10 min reaction time by addition of perchloric acid to 0.5M and the production of tritiated dCDP was measured as described (27). Thioredoxin reductase and NADPH could be substituted by 10 mM DTT in the [3H]CDP assays without change in specific enzyme activity.

The ribonucleotide reductase activity is defined in units/mg protein, which is the amount of protein R1 that converts 1 nmol of substrate per minute in the presence of an excess of R2 protein at 25 °C. The specific activity is expressed as units/mg of protein R1 or nmol/min/mg protein. Extinction coefficients were ε₂₈₀₋₃₁₀ = 18,000 M⁻¹ cm⁻¹ for protein R1 and ε₂₈₀₋₃₁₀ = 120,000 M⁻¹ cm⁻¹ for protein R2.

**Determination of R1-R2 Dissociation Constants—**The R1-R2 protein interaction was measured by the [3H]CDP assay or by surface plasmon resonance measurements. In the [3H]CDP assay the *Kd* for interaction of wild type or N238A R1 proteins with the R2 protein was determined according to the method by Climent et al. (28). The R1 protein concentration was kept constant at 0.033 μM, and the concentration of the R2

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**Table I**

| Mutation | Mutagenesis primer sequences* |
|----------|------------------------------|
| N238A    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| V245K    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| A273N    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| T276Q    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| A270N/T276Q | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| H284F    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| H284V    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |
| T287N    | 5′-GGT GAC GGC CTT ATC GCC ACC ACC GCG-3′ |

* Underlined nucleotides denotes mismatches.
 Protein was varied between 0.033 and 2 μM. $K_D$ values were calculated from plots of enzyme activity versus R2 protein concentration and non-linear curve fitting to Equation 1 (28).

$$v = \frac{V_{max}}{K_M + R2 + R1} - \frac{K_M}{2R1} \left( \frac{K_M + R2 + R1}{R1} \right)^n - \frac{R2}{R1}$$  \hspace{1cm} (Eq. 1)

In the surface plasmon resonance assay the interaction of wild type or N238A R1 proteins with the R2 protein was studied using the Amersham Biosciences Biosensor (BIACore) method. The $K_D$ of R1 protein N238A interacting with protein R2 in the presence of 100 μM of enzymatically inactive Y122F-R2 protein. All experiments were carried out as with dTTP but in the presence of 1 mM of GDP or 1 mM CDP. Binding experiments on the R1-R2 complex were performed in the presence of 7.5 μM of enzymatically inactive Y122F-R2 protein. All experiments were carried out at room temperature in Ultrafree-MC filter units with polysulfone membranes, molecular cut-off 30,000, obtained from Millipore. Labeled [methyl-3H]dTTP and cold dTTP was purchased from Amersham Biosciences (GE Healthcare). Kinetic data were obtained from a plot of the concentration of bound ligand per concentration of protein ($V$) versus concentration of free ligand ($L$). Fitting Equation 2 to the curve gives the dissociation constant ($K_D$) and number of binding sites ($n$). All calculations were performed using KaleidaGraph software (Synergy Software).

$$V = nL(K_D + L)$$  \hspace{1cm} (Eq. 2)

RESULTS

In general all mutated proteins behaved like the wild type protein in the overproduction and purification procedures and were obtained in good yields, suggesting that the mutated proteins were stable under the same conditions as the wild type protein.

**R1 α-Polypeptide Interactions in Mutant R1 Proteins**—Two complementary methods were used to compare the strength of the α-polypeptide interaction in the mutant proteins to that of wild type R1. In native polyacrylamide gel electrophoresis the electric force induces separation of weakly associated multimeric complexes and polypeptides are principally separated according to size. When loaded onto a native gel wild type R1 migrates solely as a monomer at concentrations below 1 mg/ml and in equilibrium between monomers and dimers at concentrations above 2 mg/ml. Three of the mutant R1 proteins (A273N, T276Q, and the double mutant A273N/T276Q) behaved as wild type R1 on the native gels, whereas the remaining five mutants (N238A, V245K, H284F/V, and T287N) all migrated principally as monomers even at the highest protein concentrations tested (8–20 mg/ml). Fig. 2 shows the behavior of wild type, N238A and V245K proteins, and Table II summarizes the behavior of all R1 proteins.

Gel filtration, in contrast to electrophoresis, does not impose additional forces on multimeric proteins than are imposed by the buffer conditions used for chromatography. The gel filtration analyses of wild type and mutant R1 proteins at 2.5 mg/ml protein concentration confirmed the results from native gels. The monomeric proteins did not dimerize even at the highest protein concentrations tested (8–25 mg/ml). However, two of these proteins (H284V and T287N) contained, in addition to the major distinct peak of a monomer, also material of very high molecular mass at the exclusion volume of the column indicating that these two mutant proteins had tendencies to aggregate rather than forming dimers (Fig. 3).

**Enzyme Activities of the Mutant R1 Proteins**—One helix mutation (N238A) and the loop mutations (A273N, T276Q, and A273N/T276Q) had comparatively high enzyme activities in the presence of excess R2 while the remaining proteins with mutations in the helices had low or no measurable activity at all (Table II). The most striking result of the enzyme activity measurements was that higher concentrations of mutant R1 proteins compared with wild type R1 were needed to reach a constant specific activity. Wild type RNR behaves similarly if assayed at submicromolar concentrations of R1 and R2, but constant specific activity for each wild type component (R1 or R2) is achieved in the assays if the other component (R2 or R1 in this case) is included in protein concentrations well over the dissociation constant for the R1-R2 complex (24). The behavior of the mutant R1 proteins suggests that they have a weaker interaction with protein R2 (Table II), as might be expected for R1 proteins designed with mutations at the interaction surfaces of the two α-polypeptides forming the R1 dimer.

**R1-R2 Interactions**—In general, all mutant proteins whether dimeric or monomeric, had weaker interaction with protein R2 than wild type protein R1, and in some cases, i.e. V245K and H284V, no interaction with R2 could be detected. Due to the weak complex formation the mutant proteins had accordingly lower or no enzyme activity. Interestingly, during the assay conditions used, the monomeric mutant protein N238A managed to interact with protein R2 at a weaker but sufficient strength to perform catalysis.

We therefore also performed a surface plasmon resonance assay of the R1-R2 interaction in this protein in the presence of the allosteric effector dTTP and the substrate GDP. These conditions had earlier shown tight R1-R2 interaction for wild type R1 with a decrease of dissociation constant under surface plasmon resonance conditions from −6.5 μM in the presence of only dTTP to 2 μM in the presence of both dTTP and GDP (29). Under surface plasmon resonance conditions, the mutant protein N238A had a $K_D$ of 5.8 μM in the presence of both effector
and substrate nucleotides, again showing that N238A forms a weaker complex with R2 than does wild type R1.

**Gel Filtrations of Wild Type and N238A Proteins**—To determine the oligomerization status of the N238A protein in the active holoenzyme in complex with allosteric effectors and substrate, a series of gel filtrations in the presence of effectors and/or substrate were carried out. N238A migrated as a monomer during all conditions tested except in the presence of both effector dTTP and substrate GDP when it migrated as a dimer (Fig. 4). The mutant protein V245K was used as a control in the dTTP/GDP experiment (Table II). In contrast to wild type R1 and N238A, V245K migrated as a monomer also during these conditions (Table III). In the presence of GDP the elution peak of N238A was broadened, which may indicate a low fraction of dimers. Effectors dATP and ATP have been shown to have great impact on the oligomerization of mouse R1 monomers and higher oligomeric forms ranging from dimers to hexamers have been reported (15, 18, 22, 31). In our gel filtration studies of the *E. coli* R1 protein we could not detect other forms than the dimer. Kashlan et al. have further concluded from oligomerization studies on the mouse R1 protein that the active holocomplex has a dodecameric structure (R16R26). In our preliminary gel filtrations of the *E. coli* R1 protein in the presence of protein R2, dTTP, and GDP we did not detect complexes of molecular weights corresponding to such structure (data not shown).

**Nucleotide Binding Assays**—We checked the binding of allosteric effector dTTP to N238A and wild type R1 proteins to rule out the possibility that the absence of dimerization might be due to an inability to bind effector. A comparison of the dissociation constants obtained (Table III) shows that the mutant protein had slightly decreased binding of dTTP compared with wild type. We tested what influence the protein R2 might have on dTTP binding by using an inactive mutant R2 protein (Y122F) but detected no significant effect on either of the two proteins. Binding of dTTP was increased by the presence of its cognate substrate GDP and importantly, there was no longer any difference between wild type and N238A proteins in bind-

| R1 protein | Position in structure | Concentration for dimer formation | Native PAGE | Gel filtration | $k_{cat}$ | $[^{3}H]CDP$ | SPR$^a$ |
|------------|----------------------|----------------------------------|-------------|--------------|---------|--------------|--------|
| Wild type  | aA                   | 1–1.5 mg/ml                      | 1–1.5       | 0.03 ± 0.005 | 2.0 ± 0.1 |
| N238A      | aA                   | No dimer formed$^b$              | No dimer formed$^b$ | 7.3 ± 0.5 | 5.8 ± 0.5 |
| V245K      | aA                   | No dimer formed$^b$              | No dimer formed$^b$ | >4          | ND $^+$  |
| A273N      | Loop 2               | 2–4 mg/ml                        | ≥0.025      | 3.4 ± 0.2   | 0.08 ± 0.02 | ND $^+$ |
| T276Q      | Loop 2               | 1–2 mg/ml                        | ≥0.025      | 3.6 ± 0.1   | 0.11 ± 0.01 | ND $^+$ |
| A273N/T276Q| Loop 2               | 2–4 mg/ml                        | <0.025      | 5.5 ± 0.16  | 0.19 ± 0.02 | ND $^+$ |
| H284F      | aB                   | No dimer formed$^b$              | No dimer formed$^b$ | 0.5 ± 0.03 | 0.16 ± 0.03 | ND $^+$ |
| H284V      | aB                   | No dimer formed$^b$              | No dimer formed$^b$ | No activity | >4          | ND $^+$ |
| T287N      | aB                   | No dimer formed$^b$              | No dimer formed$^b$ | 0.3 ± 0.01 | 0.05 ± 0.01 | ND $^+$ |

$^a$ Dissociation constant obtained using surface plasmon resonance. Moles of R1 protein bound per mole of R2 protein was 0.61 ± 0.06 for wt R1 and 0.55 ± 0.05 for N238A.

$^b$ The highest concentrations tested in the native gel electrophoresis and the gel filtration experiments, respectively, were 10 mg/ml (N238A), 10 and 25 mg/ml (V245K), 20 and 8 mg/ml (H284F), 8 and 12 mg/ml (H284V), and 10 and 23 mg/ml (T287N).

$^+$ ND, not determined.
The R1 protein of *E. coli* ribonucleotide reductase is known to form a tight dimer (24, 28), in contrast to R1 protein from mouse which seems to form tight dimers only when complexed with effectors (20–22, 32). The *E. coli* R1 protein used in this study is still partially dimeric at a protein concentration of 0.025 mg/ml, but monomers appear already at 0.25 mg/ml.

In the presence of nucleotides as indicated, the calculated molecular masses based on amino acid composition of monomeric and dimeric R1 proteins are 85.7 and 171.4 kDa, respectively. The varying baselines in these experiments are due to the strong UV absorption of the nucleotides, included in the running buffers.

**FIG. 4.** Gel filtration of R1 N238A and wild type R1 in the presence of effector and substrate nucleotides. In the presence of 100 μM dTTP (A) and in the presence of 100 μM dTTP plus 1 mM GDP (B). Proteins at 2.5 mg/ml were R1 wild type (black), N238A (red), and at 25 mg/ml V245K (blue). The varying baselines in these experiments are due to the strong UV absorption of the nucleotides, included in the running buffers.

**TABLE III**

| Effector       | MW N238A | MW R1 WT | MW V245K |
|----------------|----------|----------|----------|
| None           | 85       | 165      | 74       |
| DATP (100 μM)  | 85       | 157      | ND*      |
| DATP (1 μM)    | 85       | 145      | ND       |
| DATP (100 μM)  | 86       | 150      | ND       |
| ATP (1.5 mM)   | 90       | 140      | ND       |
| GDP (1 mM)     | 82       | 135      | ND       |
| dTTP/GDP       | 175      | 175      | 85       |

* ND, not determined.

**DISCUSSION**

The R1 protein of *E. coli* ribonucleotide reductase is known to form a tight dimer (24, 28), in contrast to R1 protein from mouse which seems to form tight dimers only when complexed with effectors (20–22, 32). The *E. coli* R1 protein used in this study is still partially dimeric at a protein concentration of 0.025 mg/ml, but monomers appear already at 0.25 mg/ml.

Oligomerization of the R1 protein has been suggested to play a regulatory role in mouse RNR (16). Given this apparent difference in the oligomerization status of the N238A protein in this active complex, we investigated the conditions that governed dimer formation in the N238A protein. Because binding of allosteric effectors at the specificity site is crucial for dimer formation in the mouse protein, we investigated the influence of allosteric effector nucleotides on the oligomerization status of the N238A protein. Gel filtration studies showed that the protein migrated as monomeric during all these conditions, and to make sure that this was not due to an inability to bind the effector we compared the binding of dTTP to N238A and wild type R1 proteins. The slight decrease in affinity observed in N238A was not of a magnitude that would prevent dimerization, because the concentration of dTTP used in all our assays by far exceeds the binding constant for dTTP in N238A.

**TABLE IV**

| Effector       | $K_D$, N238A | $K_D$, R1 WT |
|----------------|--------------|--------------|
| dTTP           | 1.51 ± 0.038 | 1.06 ± 0.087 |
| dTTP/GDP       | 0.48 ± 0.22  | 0.44 ± 0.19  |
| Y122F/dTTP     | 1.09 ± 0.16  | 0.87 ± 0.18  |
| Y122F/dTTP/GDP | 0.43 ± 0.07  | 0.46 ± 0.10  |

Standard errors are shown.
However, in the presence of GDP, the substrate that preferentially is reduced when dTTP is bound at the specificity site, the binding constants for dTTP in the N238A protein were the same as in the wild type protein (Table IV). The positive cooperative effect of GDP on dTTP binding observed in both proteins is well known from earlier effector binding studies (29, 30, 33, 34). Gel filtration experiments showed, in accordance with the nucleotide binding assays, that the N238A protein was able to dimerize in the presence of dTTP and GDP as it migrated identically to the wild type protein (Fig. 4). The substrate GDP alone was not fully sufficient for dimerization of the N238A protein.

In wild type R1 Asn-238 in one polypeptide is close to Ser-242 (4.14 Å from O), Val-245 (3.24 Å from CH₂), and His-284 (4.41 Å from Ne) in the other polypeptide. It is not obvious why a change from Asn to Ala would lead to such drastic effects on R1 dimer formation as seen in the N238A protein. The effects of the mutation, on the other hand, fit well with the notion that E. coli protein R1 has a comparatively small dimer interaction surface in relation to its size. When the three-dimensional structure of the dimeric E. coli R1 protein was solved the crystals showed an arrangement of three R1 dimers into a hexamer. Considering the crystallographic 2-fold axis, the dimer was regarded as the relevant form and the hexamer as a result of the crystallization procedure. However the dimeric structure of E. coli R1 protein has been questioned as experiments on mouse R1, the structure of which is not yet solved, points at higher oligomeric forms particularly in the presence of ATP (15, 18, 22, 31). In our gel filtration studies on wild type R1 protein from E. coli we have not observed other forms from the dimer either in absence of effector or in the presence of ATP, dATP, dTTP, and/or GDP (Table III) and/or a radical-less R2 protein (data not shown). We therefore conclude that the active form of RNR in E. coli is a tetrameric complex as suggested in the Uhlin and Eklund model (9). The differences between the E. coli and mouse R1 proteins thus may include not only the strength of the α₂ interaction but also the oligomerization status of R1 in the active holoenzyme.

In this study we have found that single moderate mutational changes can cause drastic effects on the quaternary structure of E. coli protein R1, which may explain why subtle differences in the interaction surfaces of R1 proteins from other species lead to proteins with weaker capacity to form dimers in the absence of effectors and/or substrates. We have identified key residues of the interaction area that also differ between prokaryotes and mammals. In analogy with treatment of hamster sarcoma virus infection where the assembly of viral but not mammalian R1 and R2 proteins can be inhibited by a R2 mimicking peptide, it may be possible to use a similar strategy to specifically inhibit the R1-R1 dimerization process in e.g. pathogenic prokaryotes.

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