Regulation of T4 Phage Aerobic Ribonucleotidase Reductase

SIMULTANEOUS ASSAY OF THE FOUR ACTIVITIES*

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Stephen P. Hendricks and Christopher K. Mathews;

From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-7305

We have devised an assay procedure that permits simultaneous monitoring of the four activities of ribonucleotide reductase. Using this assay, we have compared the reduction of all four substrates by the T4 bacteriophage aerobic ribonucleotidase reductase within different allosteric environments. Specifically, we compared the relative turnover rates by the enzyme when activated with "in vivo" concentrations of the known allosteric effectors versus activation by ATP alone. Consistent with the known allosteric properties of this enzyme, our results show that ATP does act as a general activator, although the rate of purine nucleotide reduction was approximately 5% of the rate for the pyrimidine nucleotides. However, addition of the allosteric effectors at their estimated physiological concentrations dramatically changed the relative rates of substrate reduction, creating a more "balanced" pool of products. Addition of the substrates at their respective in vivo concentrations further pushed rates of product formation toward a ratio similar to the base composition of the T4 genome. The similarity of the product profile produced under in vivo conditions to the genomic composition of T4 phage is discussed.

The first committed step in DNA biosynthesis occurs by direct reduction of the 2'-hydroxyl of ribonucleotides and is catalyzed by the enzyme ribonucleotide reductase. Because this single enzyme is responsible for the production of all four deoxyribonucleotides and because these products are needed only at specific times in cell or viral life cycles, ribonucleotide reductase is highly regulated in both substrate specificity and overall activity.

The allosteric properties of the enzyme, as well as the kinetic parameters for all four rNDP1 substrates, are well characterized for the T4 phage aerobic ribonucleotidase reductase (1) and are similar to those of the prototypical enzyme from Escherichia coli. However, to our knowledge, no studies have been done to determine what the simultaneous turnover rates for each of the substrates are under different concentrations and combinations of the enzyme's allosteric effectors and substrates. Of particular interest to our laboratory are the relative rates of formation for each of the four products when the effector and substrate environment of the enzyme mimics the in vivo conditions as measured in T4 phage-infected E. coli (2, 3). In other words, when the known allosteric effectors and substrates are supplied together in a single reaction mixture at their estimated physiological or in vivo concentrations, what are the relative rates of formation of each of the products? This question is of particular interest because, in vivo, T4 ribonucleotide reductase functions as part of an enzyme complex (4) and the possibility that intracellular reaction fluxes are controlled by protein-protein interactions must be considered.

Typically, ribonucleotide reductase has been assayed by using only one of the substrates because analysis of the simultaneous turnover of all four substrates by the enzyme was not possible with existing methods. The ability to follow the reduction of all substrates simultaneously and in a quantitative way, under varied conditions, was the goal of this method development. We describe such an assay and its application to the T4 bacteriophage aerobic ribonucleotidase reductase, in which the product profile was determined under different allosteric states.

The key requirement of this assay is the quantitative resolution of the dNDP products from the rNDP substrates and nucleoside triphosphate effectors (ATP, dATP, dGTP, and dTTP). This task is accomplished in two chromatographic steps. The first step involves separation of the deoxyribonucleotides from the ribonucleotides using boronate affinity chromatography. Molecules containing cis-diols readily bind to boronate affinity resins at basic pH by forming a complex between the vicinal alcohols of the ribose ring and the boric acid functional groups. Thus, when a mixture of ribo- and deoxyribonucleotides is applied to a boronate column the deoxyribonucleotides pass through while the ribonucleotides are retained. This type of affinity chromatography is frequently used to separate dNTPs from rNTPs derived from cell lysates when doing dNTP pool assays (5). In the second step, the boronate column eluate containing the deoxyribonucleotides is resolved into individual components by HPLC. The HPLC step uses a strong anion exchange column and an ammonium phosphate gradient to resolve the individual deoxyribonucleotides into quantifiable peaks.

**EXPERIMENTAL PROCEDURES**

**Enzyme Assay Conditions**—The enzyme assay procedure was modified from what we used previously (6). Briefly, assays were performed in 50 mM HEPES-KOH buffer (pH 8.2) containing 5 mM magnesium chloride, 50 mM dithiothreitol, and 20 μM ferrous ammonium sulfate (prepared fresh). Assays done under in vivo conditions refer to enzyme reactions carried out with the following effector and/or substrate concentrations as described by Mathews (2) and Neuhard and Nygaard (3): ATP, 2.7 μM; dATP, 175 μM; dTTP, 80 μM; dGTP, 125 μM; CDP, 80 μM; UDP, 90 μM; ADP, 250 μM; GDP, 130 μM. The concentration of the

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331-7305. Tel.: 541-737-1865; Fax: 541-737-0481; E-mail: mathewsc@ucs.orst.edu.

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holoenzyme was typically 0.25 \mu m unless otherwise indicated. The volume of each aliquot removed for assay was 100 \mu l. The reaction was stopped by addition of 5 \mu l of 50% perchloric acid. Immediately after the addition of acid, the sample was vortexed and placed on ice. After 5 min on ice, KOH was added until the pH was between 8 and 9 (6–7 m\text{M} \text{KOH}) was added to the reaction mixture. The resulting suspension was then clarified by centrifugation and the supernatant recovered and analyzed by boronate affinity chromatography followed by HPLC, as described below.

**Boronate Chromatography**—Boronate affinity chromatography was performed essentially as described by Shewach (5). Briefly, hydrated Aff-Gel 601 (Bio-Rad) was packed into a 1-ml plastic tuberculin syringe fitted with a frit. The column was equilibrated with 0.15 M ammonium bicarbonate buffer, pH 8.9, containing 15 mM magnesium chloride (buffer A). The column was fitted with a frit. The column was equilibrated with 0.15M ammonium phosphate buffer at a flow rate of 1.5 ml/min. Washing the column in buffer A. The 1.8-ml eluate was either evaporated to dryness on a Speed-vac or a 500-mlloop and then analyzed by HPLC (see Fig. 2B). As is apparent from the chromatograms, the boronate column was extremely effective at binding ribonucleotides. This is demonstrated by the absence of UDP and ATP peaks in Fig. 2B. Furthermore, comparison of the area under each of the deoxyribonucleotide peaks between the two chromatograms showed that at least 95% of each of the dNDPs was recovered after the boronate step.

To demonstrate that the method is linear with respect to enzyme concentration, assays were performed using a saturating concentration of CDP and five different enzyme concentrations ranging from 0 to 20 \mu g of protein. As Fig. 3 illustrates, the assay is linear within the range of enzyme concentrations used in this study. Using these data from the linearity experiment, we calculated the turnover rate for CDP reduction under conditions of saturating substrate to be 0.5 s\(^{-1}\). This value correlates well with the turnover rate reported by Berglund (8) when dithiothreitol is the source of reducing equivalents for the T4 ribonucleotide reductase.

**Measurement of the Simultaneous Reduction of all Four rNDP Substrates**—Fig. 4 shows the HPLC results of a typical ribonucleotide reductase assay with the reaction mixture incubated at 37 °C and sampled at 5-min intervals. In this particular experiment, the substrates and effectors were added at their in vivo concentrations, as defined under “Experimental Procedures.” As the chromatograms demonstrate, the formation of each of the four dNDP products can be followed quantitatively over time in a single reaction mixture. Fig. 5 is a graphical representation of the same data after conversion of peak areas into nanomoles of the corresponding product. Using the initial rates of product formation, we calculated the percent of total product formed for each dNDP. These data, along with the percent of total for each product formed in assays done under different effector and/or substrate concentrations, are tabulated in Table I.

**dTTP Inhibition of CDP Reduction**—The T4 aerobic ribonucleotide reductase, unlike the enzyme from *E. coli*, is considered to be relatively insensitive to allosteric inhibition (1). However, studies done in our laboratory using crude extracts from T4-infected *E. coli* have shown that CDP reductase activity is inhibited by dTTP in *situ* but that this inhibition is lost upon purification of the enzyme (9). The basis for the loss of feedback inhibition may involve a number of factors, including disruption of protein-protein interactions within the T4 enzyme complex, or, as suggested by Reichard,3 loss of ATP during the purification process. Reichard’s suggestion is based on the observation that, for the purified enzyme from *E. coli*, dTTP

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2 The rNDPs were retained by the boronate column slightly better than the rNTPs.

3 P. Reichard, personal communication.
inhibition of CDP reduction occurs only when ATP is present at intracellular concentrations (10). Using this new assay, we tested whether, in the case of the T4 enzyme, the presence of ATP was sufficient to restore feedback inhibition by dTTP in vitro. Purified enzyme was mixed with either ATP and dTTP, or ATP alone, and the reduction of CDP was followed over time. Fig. 6 shows the results of these experiments. As is evident by the similarity of the two lines, the addition of ATP does not sensitize the CDP reductase activity of the T4 enzyme to dTTP inhibition.

**DISCUSSION**

This paper presents a novel method for the simultaneous assay of all four ribonucleotide reductase activities. More importantly, using this assay, we have determined how the simultaneous rates of these individual activities are influenced by the initial substrate concentrations and the allosteric environment of the enzyme. To our knowledge, this represents the first study of this kind on ribonucleotide reductase and presents a new way of evaluating the role of the enzyme in regulating the levels of the DNA precursors.

Consistent with the known allosteric properties of the T4 aerobic ribonucleotide reductase (1), our results show that ATP does act as a general allosteric activator for all four substrates, although pyrimidine nucleotide reduction greatly exceeded reduction of the purine nucleotides. When ATP was present as the only allosteric effector and the substrates were supplied at in vivo concentration levels, the reduction of GDP and ADP represented less than 5% of the total reaction flux (Table I, row 1).

When the four ribonucleotide reductase activities were measured with the enzyme under in vivo allosteric conditions, dramatic changes in the product profile, or relative amounts of each product formed, were observed. When the in vivo levels of the nucleoside triphosphate effectors were supplied to the enzyme along with equimolar amounts of the four rNDP substrates (0.1 mM each), the percent of purine reduction increased to 35% of the total (Table I, row 2). Even more interesting were the results obtained when both the effectors and the substrates were supplied to the enzyme at their respective in vivo concentrations (Table I, row 3). Under these conditions, the amount of purine reduced increased to 43% of the total product formed, closely approaching the 50% expected in double-stranded DNA. Of the purine nucleotide reduced in this experiment, dADP represented 31% of the total product formed. This is surprisingly good agreement with the 32% adenine present in the T4 genome. The rate of formation of the other purine, dGDP, was lower than predicted from the genomic composition of guanine in T4 (12 versus 18%). This was true even though the $K_m$ for GDP is almost identical to the $K_m$ values for CDP and ADP when the prime effectors are present (1). Low GDP reduction rates may simply be an inherent property of type I ribonucleotide reductases, and this may explain, at least in part, why the dGTP pools in many cell types are underrepresented (11). It is also possible that the estimated in vivo effector and/or substrate concentrations used in these experiments do not faithfully represent the true concentrations sensed by the enzyme in vivo.

Formation of dCDP occurred at a much higher rate than that expected from its percent contribution to the T4 genome. This
may be explained by the fact that a significant portion of the dCDP produced by ribonucleotide reductase is shuttled into the production of thymine nucleotides via deamination of the cytosine base. If ribonucleotide reductase does produce products in proportions relative to the proportions seen in the genome, then we would predict from these data, since all hydroxymethylcytosine nucleotide must come from CDP reduction, that approximately one-half of the total dCDP produced under in vivo conditions, or approximately 21% of the total product produced, would be diverted into the thymine nucleotide pathway. The diverted dCDP, after the action of enzymes including dCMP deaminase, combined with the dUDP produced directly by ribonucleotide reductase makes up 35% of the total deoxyribonucleotide produced. This fits well with the 32% thymine present in the T4 genome. The flux of dCDP through dCMP deaminase predicted from these experiments suggests that roughly 65% of the total thymine pool results from cytosine deamination. This is remarkably close to thymine production in the T4 host, E. coli, in which 75% of the dTTP is derived from CDP reduction (12). The remaining portion of dCDP that is not shuttled into the thymine pathway represents 22% of the total amount of dNDP formed. The T4 genome is composed of 18% hydroxymethylcytosine. Therefore, the level of dCDP that we predict is not shuttled into thymine biosynthesis is sufficient to fulfill its contribution to the T4 genome.

The significance of this work is 2-fold. First, we describe a new assay procedure that is applicable to any ribonucleotide reductase, including the anaerobic rNTP reductases, which is relatively simple to carry out, and that permits simultaneous monitoring of the four activities of the enzyme. Since the creation of DNA precursor imbalances by drugs that specifically target ribonucleotide reductase is well-documented (13, 14) and is important clinically (15), the procedure should prove useful in evaluating therapeutically significant ribonucleotide reductase inhibitors. Second, we applied this assay to the T4 phage aerobic rNDP reductase, an enzyme whose allosteric control features are still not well understood. Berglund’s original studies on the purified enzyme suggest that it is relatively insensitive to allosteric inhibition. More recently, our laboratory showed that in vivo and in crude preparations, the CDP reductase activity of the enzyme is sensitive to dTTP, suggesting the presence of regulatory factors that might be removed during enzyme purification. The present study, carried out with purified recombinant enzyme, shows that in the presence of near physiological concentrations of substrates and effectors, the enzyme functions at relative rates that correspond closely to in vivo conditions.

TABLE I  
Relative rates of product formation by T4 ribonucleotide reductase

| Assay conditions | dCDP | dUDP | dADP | dGDP |
|------------------|------|------|------|------|
| ATP only, in vivo substrates | 85   | 11   | 4    | >1   |
| In vivo effectors, equimolar substrates | 48   | 17   | 28   | 7    |
| In vivo effectors, in vivo substrates | 43   | 14   | 31   | 12   |

* Cytosine is present in T4 bacteriophage as 5-hydroxymethylcytosine.

b 32% of the T4 genomic composition is thymine.

FIG. 4. HPLC analysis of product formation followed over time. 100-μl aliquots were removed from the assay mixture at 5-min intervals, subjected to boronate chromatography, and then analyzed by HPLC as described under “Experimental Procedures.” In this particular example the assay mixture contained in vivo concentrations of allosteric effectors and substrates.

FIG. 5. Relative rates of formation of deoxyribonucleoside diphosphates. This is a graphical representation of data from Fig. 4 after conversion of peak areas to nmol of product.

FIG. 6. dTTP inhibition of CDP reduction. Purified T4 ribonucleotide reductase was incubated at 37°C with either ATP and dTTP (2.7 mM and 200 μM, respectively) or ATP alone (2.7 mM), and the reduction of CDP was followed over time. No significant difference in the rate of CDP reduction was evident in these experiments.

![Graphical representation of data from Fig. 4](image1.png)

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* Cytosine is present in T4 bacteriophage as 5-hydroxymethylcytosine.

b 32% of the T4 genomic composition is thymine.

![Graphical representation of data from Fig. 5](image2.png)

**dTTP inhibition of CDP reduction**

Purified T4 ribonucleotide reductase was incubated at 37°C with either ATP and dTTP (2.7 mM and 200 μM, respectively) or ATP alone (2.7 mM), and the reduction of CDP was followed over time. No significant difference in the rate of CDP reduction was evident in these experiments.

![Graphical representation of data from Fig. 6](image3.png)

**dTTP inhibition of CDP reduction**

Purified T4 ribonucleotide reductase was incubated at 37°C with either ATP and dTTP (2.7 mM and 200 μM, respectively) or ATP alone (2.7 mM), and the reduction of CDP was followed over time. No significant difference in the rate of CDP reduction was evident in these experiments.
vivo reaction fluxes. Thus, while the enzyme might be regulated in part by protein-protein interactions as part of its functioning within the dNTP synthetase complex, adequate information for modulating its four activities is built into the structure of the protein itself.

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