Allosteric Regulation of the Third Ribonucleotide Reductase (NrdEF Enzyme) from Enterobacteriaceae*

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Enterobacteriaceae contain genes for three separate ribonucleotide reductases: nrdAB code for a class Ia enzyme, active during aerobiosis, nrdDG for a class III enzyme, active during anaerobiosis, and nrdEF for a cryptic class Ib enzyme. The NrdEF enzyme provides the active reductase in other, widely different bacteria. Here, we describe the allosteric regulation of the Salmonella typhimurium NrdEF enzyme. It consists of two tightly bound homodimeric proteins, R1E and R2F. Nucleoside triphosphates (ATP, dATP, dGTP, and dTTP) regulate the substrate specificity by binding to a single site of the R1E protein (one nucleotide per polypeptide). Regulation is similar to that of the NrdAB enzyme, with one major exception: dATP stimulates reduction of CDP (and UDP) under conditions when dATP strongly inhibits all activity of the NrdAB enzyme. The nrdA-coded R1 protein contains a second binding site for dATP (and ATP) that controls general enzyme activity. All known R1E proteins lack the 50 N-terminal amino acids of R1, and we propose that the activity site is located in this area of the protein. The more sophisticated regulation of NrdAB enzymes of eukaryotes provides protection against the possibly harmful overproduction of dNTPs.

The history of ribonucleotide reductases is full of surprises. A recent example is the discovery of the nrdEF genes that code for a third reductase in enterobacteriaceae (1). Two other ribonucleotide reductases were earlier known to exist in Escherichia coli: an aerobic class I enzyme, coded by the nrdAB genes (2), and the more recently found anaerobic class III enzyme, coded by nrdDG (3, 4). The third enzyme is also a class I enzyme, but its amino acid sequence and some of its properties (5) distinguish it from the previously known E. coli ribonucleotide reductase. In enterobacteriaceae, the new enzyme is poorly expressed, but recent work (6–8) has established the presence of the nrdEF genes and in some cases also the corresponding active reductase in widely different bacterial species. It now appears that in bacteria an active NrdAB-type reductase only is found in E. coli and related organisms, whereas other bacteria maintain an active NrdEF enzyme. Eukaryotes contain the NrdAB enzyme. We have suggested a subdivision of class I, with the NrdAB enzymes forming class Ia and the NrdEF enzymes forming class Ib (8).

All class I enzymes are constructed from two homodimeric proteins called R1 and R2 for class Ia and R1E and R2F for class Ib (8, 9). R1 and R1E contain substrate binding and allosteric sites, R2 and R2F contain a oxygen-linked di-iron center and a stable tyrosyl radical, required for catalytic activity. The iron center participates together with oxygen in the generation of the radical (2). Class I enzymes thus depend on molecular oxygen for their activity. R2 (R2F) provides the tyrosyl radical, but it is used by R1 (R1E) to activate the substrate that is bound in the catalytic cleft (10) and reduced by two strategically placed redox-active cysteines (10–12).

All ribonucleotide reduction involves radical chemistry and requires a protein-bound radical for substrate activation. A second group of reductases (= class II) substitutes adenosylcobalamin for R2 as radical generator (13, 14). Class II enzymes consist of a single protein that functions similar to R1 of class I enzymes. The radical generating mechanism then no longer depends on the presence of oxygen, and ribonucleotide reduction occurs equally well under anaerobic and aerobic conditions. A third group of ribonucleotide reductases (= class III) generates an oxygen-sensitive glycine radical from S-adenosylmethionine (3). Class III enzymes consist of two homodimeric proteins (4), similar to class I reductases, and again employ the small protein for radical generation. However, this protein contains an iron-sulfur cluster in place of the oxygen-linked di-iron center. Class III enzymes operate only during anaerobiosis.

All three classes of enzymes have one property in common: a single protein reduces the four common ribonucleoside di- or triphosphates (9). Substrate specificity is determined by nucleoside triphosphates acting as allosteric effectors (15–17). Thus, the presence of ATP makes the enzymes reduce CDP and UDP, dGTP favors ADP reduction, and dTTP favors GDP reduction. These effects are similar for all three classes. This is not the case for dATP. With enzymes belonging to class Ia (15), excepting virus-coded reductases, and class III (17), dATP is a general inhibitor. Enzymes belonging to class Ib (5) and II (16) are not inhibited. Instead, dATP stimulates the reduction of CDP and UDP.

The mechanism giving rise to these effects has been investigated in some detail for the E. coli class Ia (15) and III (17) reductases. In the former case, the effector nucleotides were found to bind to R1 at two distinct sites, one each per protomer. One site binds either ATP, dATP, dGTP, or dTTP and determines the specificity of the enzyme (= substrate specificity site). The second site binds only ATP or dATP and determines the overall activity of the enzyme (= activity site). With ATP bound to this site, the enzyme is active; with dATP bound, it is inactive.

Also, the large protein of class III enzymes contains two distinct effector binding sites (17). One binds dGTP, dTTP, and dATP and regulates the reduction of purine substrates, the second binds dATP and ATP and regulates pyrimidine substrates. At both sites, dATP binding turns off the enzyme.

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while binding of the other nucleotides is activating: ATP stimulates CDP and UDP reduction, dGTP stimulates ADP reduction, and dTTP stimulates GDP reduction. The physiological net effect is thus the same as with class Ia enzymes.

As mentioned above, class Ib enzymes behave differently from class Ia and III enzymes in that they are not inhibited by dATP. Here, we study the Salmonella typhimurium class Ib reductase (5) to determine the reason for this. We demonstrate that this enzyme also reduces the four common ribonucleotides and that its substrate specificity is allosterically regulated. Our results suggest that the lack of inhibition by dATP is due to the absence of an activity site that can bind this nucleotide.

**EXPERIMENTAL PROCEDURES**

**Materials**—The large (R1E) and small (R2F) proteins of the S. typhimurium Ib reductase were purified to apparent homogeneity as described (5). 3H- and 14C-Labeled nucleoside di- and triphosphates were obtained from Amersham Corp. or Moravek Biochemicals. They were diluted to a specific activity of between 5 and 10 mCi/mmol with the corresponding unlabeled nucleotides and purified by chromatography on DEAE-Sephadex (17). Unlabeled nucleoside triphosphates were obtained from Pharmacia Biotech Inc. and used without further purification except for ATP. Since high concentrations of ATP were used, it was necessary to exclude also very minor contamination with dATP. To this end, a 50 mM solution of ATP in 1 M acetic acid was heated in a boiling water bath for 5 min. This treatment completely removed adenine from dATP but left ATP relatively intact. The acid was removed by evaporation in a vacuum, and the remaining ATP was purified by chromatography on DEAE-Sephadex.

Sucrose Gradient Centrifugations—5–20% linear sucrose gradients with a total volume of 4.6 ml were prepared in 50 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 1 mM dithiothreitol (18). In one experiment, nucleoside triphosphates (100 μM dATP, dGTP, or dTTP or 2 mM ATP) were also included. Proteins in a total volume of 0.2 ml were layered on top of the gradients and centrifuged in a SW 50 Beckman rotor at 30,000 rpm and 20 °C for 15 h. Catalase (20 μg/tube, s20,w = 11.4 S) was added as an internal marker. At the end of the run, the tubes were punctured at the bottom, and fractions were collected and analyzed as described earlier (18).

**Enzyme Activity Experiments**—CDP reduction was measured by the standard method used in this laboratory (5). The reduction of ADP, GDP, and UDP was determined by the modified procedure (16) of Kim et al. (19). In all experiments, R2F was used in excess of R1E (1 to 1.5 μg of each protein). Incubations were at 37 °C for 20 min. One unit of enzyme activity is 1 nmol of product formed during 1 min.

**Nucleotide Binding Experiments**—The method of Ormø and Sjöberg (20) was used. Experiments were done either at +25 °C or +4 °C. In the latter case, all manipulations including the centrifugation step were made in a cold room. The enzyme was diluted to the desired concentration (0.1 to 1.2 mg/ml) in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 2 mM dithiothreitol (0.1 ml per experiment) and added to 0.05 ml containing the radioactive nucleotide at 1 to 30 μM in a Millipore Ultrafree-MC 30000 NMWL filter unit. A 20-μl portion was withdrawn to determine total radioactivity. After 10 min, the unit was centrifuged for 3 min at 3300 rpm in an Ole Dich type 157MP centrifuge. A 40-μl portion of the filtrate (total volume approximately 50 μl) was used to determine the radioactivity of the unbound nucleotide. The amount of bound nucleotide was obtained from the difference between the total and unbound radioactivity.

**Protein Determinations**—The method (21) was first standardized by amino acid analysis of pure R1E and R2F, respectively. Thereafter, crystalline bovine serum albumin was routinely used as standard.

**RESULTS**

The Reductase Has an α2β2 Structure—The reduction of CDP required the simultaneous presence of two proteins that were separated by DEAE-chromatography early during purification of the enzyme (5). They were named R1E and R2F because of their relatedness to the large (R1) and small (R2) proteins of the earlier known aerobic E. coli reductase. This enzyme has an α2β2 structure formed from the homodimeric R1 and R2 proteins. We now show by sucrose gradient centrifugation that also the new reductase has such a structure.

In the experiment described in Fig. 1, solutions of R1E and R2F, either alone or in combination, were centrifuged in separate tubes containing 5 to 20% sucrose gradients as described under "Experimental Procedures." The s20,w values of the proteins were calculated from their positions in the gradient relative to a catalase marker (11.4 S). When alone (Fig. 1A), the two proteins migrated as symmetrical peaks with values of 8.2 S (R1E) and 4.8 S (R2F), as compared to reported values of 7.8 and 5.5 S for R1 and R2, respectively. In a parallel run, aldolase (M, = 158,000) and bovine serum albumin (M, = 67,000) gave values of 7.9 S and 4.8 S, respectively (data not shown). Taken together, these results strongly suggest that both R1E and R2F were present as dimers in solution since their monomeric molecular weights are 80,500 and 36,000, respectively.

To study complex formation, we centrifuged mixtures of the two proteins, containing increasing amounts of R2F in the presence of a constant amount of R1E (Fig. 1, B–D). At the lowest concentration of R2F, with a R2F/R1E ratio of 0.9 (panel B), a single peak was found at 9.6 S, indicating complex formation. When the ratio was increased to 1.3 (panel C), an additional peak was found at 5 S reflecting the excess R2F in the gradient. Increasing the ratio to 1.6 (panel D) also increased the amount of R2F. These results demonstrate that R1E became saturated with R2F when the stoichiometry between the two proteins was 1:1 and indicate that the complex had an α2β2 structure.

Qualitatively, these gradients look similar to those obtained earlier with R1-R2 (18). One difference concerns the stability of
The reduction of the four ribonucleoside diphosphate substrates (0.5 mM) was determined in separate experiments in the presence of the various indicated effector ribonucleoside triphosphates (50 mM for deoxynucleotides, 2 mM for ATP) as indicated under "Experimental Procedures." The activities obtained with the best effector are given as 100% and correspond to 0.18 unit for ADP, 0.17 for CDP, 0.14 for GDP, and 0.10 for UDP.

| Substrate | None | ATP | dATP | dCTP | dGTP | dTTP |
|-----------|------|-----|------|------|------|------|
| ADP       | 0    | ND  | 7    | 0    | 100  | 3    |
| GDP       | 0    | 17  | 24   | 18   | 39   | 100  |
| CDP       | 10   | 60  | 100  | ND   | 30   | 72   |
| UDP       | 20   | 90  | 100  | ND   | 45   | 50   |

* ND = not done.

The reduction from linearity (see below), $K_m$

Since the curves for the determination of reduction were optimal with dTTP, but also other effectors required concentrations of the nucleotides that were at least 10% active. Furthermore, the activity with dTTP and dATP was lower in the absence of effectors. ADP reduction showed a very specific effect on the effector giving the highest reduction.

The two pyrimidine substrates were not reduced at all in the absence of effectors. ADP reduction showed a very specific requirement for dGTP, with dTTP and dATP being less than 10% active. Furthermore, the activity with dTTP and dATP required concentrations of the nucleotides that were at least 10-fold higher than the optimal concentration of dGTP. GDP reduction was optimal with dTTP, but also other effectors showed some activity, again, however, at considerably higher concentrations.

The two purine substrates were not reduced at all in the absence of effectors. ATP reduction showed a very specific requirement for dGTP, with dTTP and dATP being less than 10% active. Furthermore, the activity with dTTP and dATP required concentrations of the nucleotides that were at least 10-fold higher than the optimal concentration of dGTP. GDP reduction was optimal with dTTP, but also other effectors showed some activity, again, however, at considerably higher concentrations.

The two pyrimidine substrates had similar effector requirements. Both showed a basal reduction in the absence of effectors. The effectors decreased the $K_m$ value for the substrate. The $K_m$ for CDP was 330 μM in the absence of effector and 20 μM in the presence of dATP (data not shown). Both pyrimidine substrates showed the best activity with dATP ("$K_m$" for CDP reduction = 2.5 μM), followed by dTTP ("$K_m$" = 60 μM) and dGTP ("$K_m$" = 60 μM) (data not shown). Since the curves for the determination of $K_m$ deviated slightly from linearity (see below), $K_m$ is put between quotation marks.

These results are similar to those found earlier for the E. coli class IIA reductase, with the exception of dATP. Here, dATP acts as a positive effector and is actually the preferred one for the reduction of pyrimidine ribonucleotides. With the class IIA and III reductases, dATP inhibited the reduction of all ribonucleosides.

Inhibition by Simultaneous Presence of Effectors—To understand the mechanism through which a given effector promotes the reduction of a specific substrate, we tested the influence of dTTP or dATP on the reduction of ADP in the presence of dGTP. As shown in Table I, dGTP is by far the best effector for the reduction of ADP, but dATP and dTTP also show minor stimulatory effects. In the experiment depicted in Fig. 2, we measured the dependence of ADP reduction on the concentration of dGTP, either alone or in the presence of a fixed amount of dATP or dTTP. The data are represented as Lineweaver-Burk plots. With dGTP alone, the curve is nonlinear with a slight upward bend indicating cooperativity. This behavior was found consistently in three separate experiments. It is not possible to calculate a $K_m$ value from such a curve. As a substitute, we measured the concentration of dGTP at half-maximal velocity. This value is 5 μM with dGTP alone and 10 μM in the presence of either 50 μM dATP or 100 μM dTTP. The three curves intersect at the ordinates (Fig. 2) indicating competition between dGTP and dATP (dTTP) for the same site on the enzyme. We will return to this experiment under "Discussion."

Binding of Nucleoside Triphosphates to Reductase—Binding of dATP, dGTP, and dTTP to R1E could be demonstrated and quantitated, whereas ATP binding was too weak to be measured. R2F alone did not bind any nucleotide but, as shown below, influenced binding to R1E.

In Fig. 3, data for binding of the three deoxynucleoside triphosphates are given as Scatchard (22) plots. Panels A–C show the binding of dATP, dTTP, and dGTP to R1E alone, panel D shows the binding of dGTP to R1E in the presence of an excess of R2F. For dATP and dGTP, binding curves at both 4 and 25 °C are given in panels B and C, respectively. Binding of dTTP (panel A) was too weak to permit experiments at the higher temperature. The following general conclusions can be drawn from the results: (i) the curves are nonlinear, indicating cooperativity; (ii) binding is 3 to 4 times stronger at the lower temperature; (iii) dGTP shows the highest affinity, followed by dATP, followed by dTTP; (iv) at least for dGTP, the presence of R2F decreases the affinity of R1E for the nucleotide; (v) extrapolation of the curves to the abscissa gives in all cases a number of approximately 2 molecules of dNTP bound per dimer of R1E.

We next asked the question whether the different nucleotides occupy the same binding site. We carried out two sets of experiments, both measuring competition between nucleotides for binding to R1E alone. In the first, we chased bound dATP from the enzyme with an excess of a competing nucleotide; in the second we determined the effect of the competing nucleotide on the binding of increasing concentrations of dGTP.

In the first experiment, a main objective was to determine if the competing nucleotide could chase dATP completely from both binding sites. The data shown in Table II show that this is the case for dGTP and dTTP, but not for ATP. ATP only moderately decreased dATP binding, even though the concentration of ATP was 100-fold higher than that of dATP. This is however explained by the very low affinity of the enzyme for ATP as shown in the next experiment.

This experiment (Fig. 4) clearly demonstrates competition of dATP, dTTP, and ATP for dGTP binding. The data are given as reciprocal plots of dGTP concentration versus binding of dGTP in order to show that increasing concentrations of dGTP over-
come the inhibition by each of the nucleotides. In this case, ATP also gave a competitive behavior.

The cooperative binding behavior prevents a designation of simple $K_{diss}$ values for the various effectors. Estimates of the values at which half of the sites are occupied give the following $\mu$M concentrations: $dGTP = 0.12; dATP = 1; dTTP = 3; ATP = approximately 500.$

**DISCUSSION**

In enterobacteriaceae the function of the NrdEF enzymes is not known. In these bacteria, the NrdAB enzymes are exclusively responsible for deoxyribonucleotide production, and conditional mutations in the $nrdAB$ genes are not complemented by the resident chromosomal $nrdEF$ genes. Both $E. coli$ and $S. typhimurium$ have maintained in their genomes the potential to produce active NrdEF proteins, apparently without using it. However, NrdEF enzymes are used for the production of deoxyribonucleotides by other bacteria (6–8). The characterization of these enzymes and an understanding of the differences between the two subgroups of class I reductases is therefore of considerable interest.

The present work concerns the substrate specificity and allosteric properties of the $S. typhimurium$ NrdEF proteins and by extrapolation those of other class I ribonucleotidereductases. We show that a single enzyme is able to catalyze the reduction of each of the four common ribonucleotides and that its specificity is modulated by nucleoside triphosphate. The enzyme is a ribonucleoside diphosphate reductase, and, thus, modulators and substrates have different levels of phosphorylation. This also applies to class Ia enzymes, but not to most class II and class III enzymes, where both substrates and modulators are triphosphates. In the present case, there can be little doubt that the modulators bind to sites on the enzyme that are distinct from the substrate binding site and that the

**FIG. 2.** Competitive inhibition of ADP reduction by $dTTP$ and $dATP$. The dependence of ADP reduction on $dGTP$ concentration was measured directly (+) or in the presence of 50 $\mu$M $dATP$ (x) or 100 $\mu$M $dTTP$ (o). The data are given as a Lineweaver-Burk plot in which the inverted rate of the reaction (1/$v$) is plotted against the inverted concentration of $dGTP$ (1/$m$M). The three curves extrapolate to the same intersection on the abscissa indicating competition between $dGTP$ and $dATP$ and $dTTP$.

**FIG. 3.** Scatchard plots for binding of effectors. A–C show binding of $dTTP$ (A), $dATP$ (B), and $dGTP$ (C) to R1E alone, D shows binding of $dGTP$ to R1E in the presence of a 1.5 molar excess of R2F. In B to D, binding is shown at two temperatures (4 and 25°C). In all curves, the abscissa shows the number of binding sites per dimer R1E whereas the ordinate gives the number of sites divided by the substrate concentration ( $\mu$M). All curves extrapolate approximately to 2 sites per dimer, and most of them show cooperative behavior. Binding at 25°C (filled circles) is approximately 4 times less than at 4°C (+).

**TABLE II**

| Addition ($\mu$M) | Molecules dATP/dimer R1E |
|------------------|------------------------|
| None             | 1.7                    |
| dGTP (13)        | 0.16                   |
| dGTP (66)        | 0                      |
| dTTP (33)        | 0.77                   |
| dTTP (133)       | 0.29                   |
| ATP (133)        | 1.6                    |
| ATP (670)        | 1.2                    |

**FIG. 4.** Competition between $dATP$, $dTTP$, or ATP for $dGTP$ binding to R1E. The results are presented similarly to Fig. 2 and show on the ordinate the inverted number of molecules of bound $dGTP$ per dimer R1E and at the abscissa the inverted concentration of $dGTP$. Extrapolation to the ordinate gives the number of binding sites for $dGTP$. The four curves compare the binding of $dGTP$ alone (+) with the binding of $dGTP$ in the presence of 7 $\mu$M $dATP$ (C), 14 $\mu$M $dTTP$ (●), or 670 $\mu$M ATP (x). All curves extrapolate to a value of 0.4 corresponding to 2.5 binding sites demonstrating that high $dGTP$ concentrations overcome the inhibition by each of the three competing nucleotides. The concavity of the curves indicates cooperativity between the two sites.
observed changes in substrate specificity are of an allosteric nature. They probably occur through subtle conformational changes at that part of the catalytic site that is responsible for the binding of the purine and pyrimidine moiety of the substrate. Thus, binding of dGTP to the allosteric site induces at the substrate binding site the appropriate conformation to bind the adenine moiety of ADP, dTPP binding gives the best fit for the guanine moiety of GDP, and dATP (ATP) binding the best fit for the pyrimidine of CDP or UDP. This model is strongly supported by the results of Fig. 2 that demonstrate a competitive inhibition of the positive effect of dGTP on ADP reduction by dTPP or ATP. The latter two effectors induce conformations of the enzyme that favor GDP or CDP reduction and therefore suppress the reduction of ADP.

It is a remarkable fact that on the whole the same results are found with all three classes of reductases, even though their amino acid sequences show little homology. The close similarity in allosteric behavior points to a common ancestor from which all ribonucleotide reductases have inherited the mechanism for the regulation of substrate specificity (9). Its presence in all classes indicates that this allosteric mechanism was invented early during evolution.

One difference between class Ia and Ib concerns the effect of dATP. In both cases, low concentrations of dATP stimulate the reduction of pyrimidine ribonucleotides. However, increasing the concentration of dATP above 1 μM inhibits class Ia (15) but not class Ib enzymes. Inhibition depends on the presence of a second binding site for dATP (activity site). One R1 polypeptide of class Ia can bind two molecules of dATP, each with different affinities, and binding of the second dATP inhibits the enzyme. We now find that one R1 polypeptide of class Ib only can bind one dATP and that this binding occurs at the substrate specificity site. Thus, class Ib lacks the activity site of class Ia.

The R1E proteins from all class Ib reductases known so far lack the 50 to 60 N-terminal amino acids of the R1 proteins of class Ia. We suggest that the activity site of class Ia is located in this part of the molecule. In support of this is the finding that a D57N mutation in the mouse R1 protein makes the enzyme insensitive to inhibition by dATP (23).

Fig. 5 shows our model for effector binding to the R1E protein of class Ib. Each protein monomer has only one binding site, and all effectors (dGTP, dATP, dTPP, and ATP) bind to this identical site. This becomes evident from the competition experiments of Figs. 2 and 5 and Table II. dNTPs are bound with a much higher affinity than ATP, the difference between dGTP (highest affinity) and ATP being more than 1000-fold. Binding is stronger at lower temperature and in the absence of the R2F protein. Binding is cooperative as indicated from the curvatures of the Scatchard plots. The explanation for this is probably that R1E as a dimer binds two molecules of effector and that binding of the first molecule increases the affinity for the second molecule. Also shown in Fig. 5 are the earlier proposed models for R1 (class Ia) and for the large protein of the anaerobic class III E. coli reductase (15, 17). Both have two allosteric binding sites per monomer. R1 has one site that can accommodate only ATP or dATP, and binding of dATP to this site inhibits the enzyme, whereas ATP binding stimulates it. We have therefore called this site activity site. It is absent from R1E. The second site on R1 that binds ATP, dATP, dGTP, and dTPP corresponds to the single binding site on R1E and regulates the substrate specificity.

None of the two binding sites of the large protein of the anaerobic class III reductase is an “activity site” in the sense of the R1 protein. dATP is a negative effector when bound to either site, but ATP counteracts its effect only at one of them (pyrimidine site). At the other (purine site), dTPP and dGTP, but not ATP, compete with dATP (Fig. 5). The final outcome is nevertheless that dATP is a general negative effector of the enzyme.

The difference in binding behavior explains why class Ib enzymes are not inhibited by dATP and therefore lack a general negative control of ribonucleotide reduction that turns off the enzyme when dNTPs accumulate. Also, class II enzymes do not have this type of control which therefore does not exist in most prokaryotes. Eukaryotic cells employ class Ia enzymes and therefore have a more sophisticated ribonucleotide reductase that protects them from the possible toxic and mutagenic effects that may arise from the overproduction of dATP and other dNTPs (24).

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