Activation of Class III Ribonucleotide Reductase by Thioredoxin*

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Anaerobic ribonucleotide reductase provides facultative and obligate anaerobic microorganisms with the deoxyribonucleoside triphosphates used for DNA chain elongation and repair. In Escherichia coli, the dimeric a2 enzyme contains, in its active form, a glycyl radical essential for the reduction of the substrate. The introduction of the glycyl radical results from the reductive cleavage of S-adenosylmethionine catalyzed by the reduced (4Fe-4S) center of a small activating protein called b. This activation reaction has long been known to have an absolute requirement for dithiothreitol. Here, we report that thioredoxin, along with NADPH and NAPDH:thioredoxin oxidoreductase, efficiently replaces dithiothreitol and reduces an unsuspected critical disulfide bond probably located on the C terminus of the a protein. Activation of reduced a protein does not require dithiothreitol or thioredoxin anymore, and activation rates are much faster than previously reported. Thus, in E. coli, thioredoxin has very different roles for class I ribonucleotide reductase where it is required for the substrate turnover and class III ribonucleotide reductase where it acts only for the activation of the enzyme.

Class III ribonucleotide reductases (RNRs)† are found in anaerobic bacteria where they supply the cell with the dNTPs needed for DNA chain elongation and repair (1). The dNTPs are obtained by direct reduction of their corresponding ribonucleotides in a reaction basically similar to the three RNR classes and initiated by hydrogen abstraction at the C3′ ribose substrate by a cysteinyl radical (2). The cysteinyl radical itself is derived from a stable protein radical (a tyrosyl radical in class I and a glycyl radical in class III) or from an organometallic cofactor (class II).

In the anaerobic class III enzyme from Escherichia coli, the protein radical is located on the polypeptide backbone at the Gly681 residue of the dimeric a2 (2 × 80 kDa) protein (3). The glycyl radical (Gly′) is formed by the concerted action of the following four components: (i) a reducing system consisting of NADPH, flavodoxin, and NAPDH:flavodoxin reductase (4, 5); (ii) a 17.5-kDa iron-sulfur protein called b or “activase” (6, 7) whose function is to catalyze the reductive cleavage of (iii) an acceptor molecule identified as S-adenosylmethionine (AdoMet) (8, 9). The reaction also requires (iv) dithiothreitol (DTT), a nonphysiological reductant (10). In the inactive resting state, the two proteins a and b forms a tight a2b2 complex, but under the reducing conditions leading to the introduction of the radical (the activation reaction), the small b protein is able to activate several molecules of the a protein (6, 7).

A second characteristic sets class III apart from the two other classes. In class III enzymes the electrons needed for the reduction of the ribonucleotides are provided by formate (11, 12). On the contrary, in classes I and II, these electrons are supplied by NADPH through thioredoxin or glutaredoxin (13).

In this paper, we show that the thioredoxin system efficiently replaces DTT during activation of class III RNR. Our data suggest that this system keeps the conserved cysteines of the C terminus of the a polypeptide in a reduced form needed for radical generation. This result solves an intriguing question concerning the function of DTT and the identity of its unknown physiological counterpart.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes and other components of the anaerobic ribonucleotide reductase system have been obtained as previously described (10, 14). Thioredoxin and thioredoxin reductase from E. coli, Chlamydomonas reinhardtii, and Arabidopsis thaliana were provided by Dr. J. Cozzis (Grenoble, France) and J. P. Jacquot (Nancy, France). AdoMet and S-adenosylhomocysteine (SAH) were purchased from Roche Diagnostics.

**Enzyme Assay—**In the first activating step, the a protein (1.2 μg), in a total volume of 35 μl, was incubated on a manifold for 60 min at room temperature under a flux of moist argon with DTT (0.5–8 mM) or TTX (5–10 mM) and the activation mix containing 55 mM Tris-HCl, pH 8.0, 55 mM KCl, 3.4 mM NAPDH, 130 μM AdoMet, 0.1 μM of the b protein, 0.5 μM flavodoxin, 0.15 μM flavodoxin oxidoreductase, and 0.1 μM thioredoxin oxidoreductase. The latter was omitted in the standard (DTT) assay. In the second step, 15 μl of the substrate mixture (giving a final concentration of 1.4 mM [3H]CTP (20–30 cpm/pmol), 1 mM ATP, 10 mM MgCl2, 10 mM HCOONa, and 100 μM EDTA) was added to initiate the reduction of the substrate. When no interference from further activation was detected, 200 μM SAH (final concentration) was included in the substrate mix. The reaction was stopped after 20 min by opening the tubes to air and addition of 0.5 ml of 1 M HClO4. The solution was then worked up as described earlier (14). One unit of enzyme activity is defined as the formation of 1 nmol of dCTP per min.

**Preparation of the Reduced a Protein—**The a protein (820 μg) was incubated with TRX (5.1 μg), TRX (1.3 μg), and NAPDH (1.6 mM) in 0.1 M Tris-HCl, 50 mM KCl, pH 8.0. Oxidation of NADPH was monitored at 340 nm inside the glove box by UV-visible spectroscopy. After 2 h, the solution was diluted 20-fold with buffer and loaded on a dATP-Sepharose affinity column (2 ml) at 0.2 ml/min⁻¹. The column was washed with 10 ml of buffer, and the reduced a protein was eluted with buffer.
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RESULTS

Assay of the anaerobic reductase activity is routinely measured by the formation of dCTP. The α protein is first activated under anaerobic conditions by a 1-h incubation with a source of electrons (the flavodoxin system or chemical reductants), AdoMet, and the activating β protein (6). This step is absolutely dependent on the presence of small thiol molecules, most effective being DTT (10). In a second step, the reduction step, the activated enzyme is incubated with the CTP substrate, the positive effector ATP, Mg²⁺, and formate. SAH can be added to this step to allow for the reduction to proceed in the absence of further activation (16). This standard assay was here modified by addition of 100 μM EDTA in the buffer as this greatly stimulated the activity.²

As shown in Fig. 1 we demonstrated that, with catalytic thiol oxidoreductase and NADPH, thioreredoxin was, at micromolar concentration, at least as efficient as DTT in the millimolar range for enzyme activity. TRXs from different sources (E. coli and A. thaliana) were found equally effective (not shown). A $K_{\text{m(app)}}$ of $0.8 \pm 0.3$ μM for TRX from E. coli in this reaction has been determined (Fig. 1, inset). Moreover the effect of TRX on the activity was restricted to the activation step only because there was almost no activity when TRX was added in the reduction step along with SAH. This behavior closely resembled that observed with DTT. No TRX dependent activity could be observed in the absence of TRR.

In Fig. 2 is shown the time-dependent decrease of the absorbance of NADPH (1.6 mM) at 340 nm when a solution of the α protein (820 μM) was complemented inside the glove box first with TRR (1.3 μM) and after 10 min with TRX (5.7 μM). Control experiments done without protein α showed a negligible decrease of the absorbance at 340 nm compared with that seen in Fig. 2. Quantitative analysis of these data showed that the reaction was almost completed after 90 min when about 820 μM of NADPH has been converted to NADP⁺.

This amount exactly matched the concentration of the α polypeptide. This indicated that the protein α had enjoyed a 2-electron reduction, and because this reaction is catalyzed by TRX it also strongly suggested that, in the as-isolated form, protein α carries a disulfide group amenable to reduction. Taken together these experiments suggest that the activation of the anaerobic ribonucleotide reductase is dependent on the presence of cysteine-free SH groups on protein α.

Reduced protein α, after treatment with the TRX system as depicted in Fig. 2, was isolated by chromatography on an affinity dATP-Sepharose column inside the glove box. After

FIG. 1. DTT and TRX concentration dependence of CTP reductase activity. The α protein (1.25 μg) was incubated anaerobically for 1 h at room temperature with the activation mix as described under “Experimental Procedures” in the absence (▲) or in the presence of DTT (●) or TRX (●). The tubes containing the protein α alone (▲) were then supplemented with TRX just before adding the substrate mix containing SAH (200 μM) to all tubes. Inset, Lineweaver-Burk plot with varying concentrations of TRX. The experimental values were fitted to a linear regression program giving a correlation factor $r = 0.997$ (Kaleidagraph).

FIG. 2. TRX-mediated oxidation of NADPH by the α protein. In a 1-mm optical path UV-visible cuvette, the α protein (820 μM) was incubated inside the glove box with NADPH (1.6 mM) and thioreredoxin reductase (1.3 μM) in 0.1 M Tris-HCl, 50 mM KCl, pH 8.0. After 11 min, TRX (5.7 μM) was added, and the reaction was allowed to proceed for 90 min. Containing ATP (1 mM). The reduced α protein was then repetitively concentrated with an ATP-free buffer to a final concentration of 10–20 mg/ml column.

FIG. 3. CTP reductase activity of the α protein. The α protein reduced and assayed without DTT (●), as isolated and assayed without DTT (●), and as isolated and assayed in the presence of 5 mM DTT (●).

FIG. 4. Time course for the activation of protein α. The reduced α protein was incubated for the indicated times with the activation mix as described under “Experimental Procedures,” and the level of activation was measured at time intervals by the CTP reductase activity of an aliquot of the mixture.

² Unpublished results.
washing the column with 5 volumes of buffer, the reduced protein was eluted with the admission of 1 mM ATP in buffer. It was then assayed for its CTP reductase activity. In Fig. 3 is compared the activity of the reduced protein \( \alpha \) isolated after dATP-Sepharose with that of the as-oxidized (oxidized) protein \( \alpha \) in the presence or in the absence of DTT. The activity was linearly related to the amount of enzyme except for the data at high protein concentration, which may be explained by the near exhaustion of the substrate. In agreement with previous data, the oxidized \( \alpha \) protein alone was found completely inactive and required DTT for activity (10). On the other hand, the reduced \( \alpha \) protein was found active in the absence of any additional reductant and displayed the highest enzyme activity. The same results essentially were obtained when protein \( \alpha \) was treated with DTT and isolated by Sephadex G-25 chromatography under strict anaerobic conditions (data not shown). However, in that case, reduction of protein \( \alpha \) required a higher concentration of reductant (100 mM) and long incubation time.

Our data show that reduction of protein \( \alpha \) either by DTT or TRX is a rather slow process. Accordingly, in previous studies using DTT and oxidized preparations of protein \( \alpha \), activation of the enzyme required prolonged incubation (at least 30–45 min) (8, 9, 14). However, as shown in Fig. 4, using reduced protein \( \alpha \) in the absence of DTT or TRX, activation occurred at a much faster rate, because about 50% of the maximal activity was achieved in less than 1 min. It is thus very likely that in the previously reported studies, enzyme activation was in fact rate-limited by reduction of protein \( \alpha \).

**DISCUSSION**

Cysteines are central to the activity of class I and II ribonucleotide reductases (2). They participate either as a thiol for initiating substrate reduction or as a dithiol in the subsequent steps leading to the deoxyribose product (2, 17). The latter cysteine pair is maintained in the reduced form by thioredoxin or glutaredoxin (13). In class III RNR, alignments of the 29 nrdD sequences presently available have revealed, among the 15–20 Cys generally present, the occurrence of five invariants, one highly conserved and three moderately conserved cysteine residues (18). Site-directed mutagenesis studies done on the enzyme from the bacteriophage T4 have demonstrated that two of these invariants are directly involved in the turnover of the reaction in agreement with the presence of two cysteines in the substrate site observed in the three-dimensional structure of the protein (12, 19). The three other invariants are part of a CXXC\(_X\)CXXC motif (not visible in the three-dimensional structure) located in the C terminus of the polypeptide. Each of these cysteines was essential for the formation of Gly' and was thus proposed to participate to radical transfer reactions during enzyme activation (19).

Activation of class III RNR has long been known to be a slow process and to rely upon the obligate presence of DTT in the activation mix (14). However, nothing was known about the function of DTT or the nature of its physiological counterpart. The data reported here strongly suggest that the role of DTT was to reduce an unsuspected disulfide on protein \( \alpha \). From the study of Andersson et al. (19), it is likely that this disulfide, in the E. coli enzyme, is located on the Cys\(_{562,665}\) pair, but this has to be confirmed with appropriate mutants. This disulfide can be reduced by the TRX/TRR system resulting in a fully active enzyme. As a consequence, reduced protein \( \alpha \) can now be activated in a fast reaction that does not require DTT or the TRX/TRR system anymore.

It thus seems that, in previous studies with the oxidized preparations, protein \( \alpha \) activation not only resided in the introduction of the glycyl radical but also implicated the reduction of important cysteines by DTT. The fact that the activation reaction becomes much faster with reduced protein \( \alpha \) (Fig. 4) indicates that the rate-limiting step during DTT-dependent activation reaction was not the generation of Gly' by itself but instead the reduction of protein \( \alpha \).

At this stage it is difficult to know the exact function of the thiols in the C terminus of protein \( \alpha \). They may have a structural role in the binding of protein \( \beta \) and/or AdoMet. They also may be involved in a radical chain transfer from AdoMet, supposed to bind at the interface of the two proteins \( \alpha \) and \( \beta \), to the glycine residue on protein \( \alpha \) as proposed by Andersson et al. (19). Finally they could be directly involved in the cleavage of AdoMet. Our recent finding that the latter reaction is triggered by addition of DTT to the (4Fe-4S)\(^{2+}\)/AdoMet complex on one hand and that DTT binds to the cluster in the other makes this third alternative very attractive (8).

It is well established now that very few cystine pairs survive the reducing conditions existing inside the anaerobic cell (20, 21). In addition to the redoxin systems, E. coli contains a high concentration of GSH, itself able to sustain CTP reductase activity *in vitro* (not shown). So, *in vivo*, protein \( \alpha \) is likely to have all of its cysteines in their reduced form providing conditions for a rapid activation. The requirement for DTT or TRX *in vitro* might just be a consequence of the isolation and oxygen sensitivity of the protein. The study of the activation reaction conducted with reduced protein \( \alpha \) is expected to solve many of the questions still unanswered.

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3 Unpublished results.
