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Biochemistry

Formate is the hydrogen donor for the anaerobic ribonucleotide reductase from *Escherichia coli*

(deoxyribonucleotide synthesis/glycyl radical/iron–sulfur center/evolution)

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ABSTRACT **During anaerobic growth *Escherichia coli* uses a specific ribonucleoside-triphosphate reductase (class III enzyme) for the production of deoxyribonucleoside triphosphates. In its active form, the enzyme contains an iron–sulfur center and an oxygen-sensitive glycyl radical (Gly-681). The radical is generated in the inactive protein from *S*-adenosylmethionine by an auxiliary enzyme system present in *E. coli*. By modification of the previous purification procedure, we now prepared a glycyl radical-containing reductase, active in the absence of the auxiliary reducing enzyme system. This reductase uses formate as hydrogen donor in the reaction. During catalysis, formate is stoichiometrically oxidized to CO_2, and isotope from [^{3}H]formate appears in water. Thus *E. coli* uses completely different hydrogen donors for the reduction of ribonucleotides during anaerobic and aerobic growth. The aerobic class I reductase employs redox-active thiols from thioredoxin or glutaredoxin to this purpose. The present results strengthen speculations that class III enzymes arose early during the evolution of DNA.**

Ribonucleotide reductases are a group of enzymes that provide the deoxyribonucleoside triphosphates required for DNA synthesis. Surprisingly, three different classes of reductases have been described, each with a distinct protein structure (1). At the substrate level, all enzymes apparently use identical radical chemistry for the reduction of C-2′ of the ribose but employ widely different mechanisms to produce the required protein radical (2, 3). Class I enzymes, as exemplified by the aerobic *Escherichia coli* reductase, contain a diferric oxygen-linked iron center and a stable tyrosyl radical (4) that during the catalytic reaction generates a transient cysteinyl radical by intramolecular electron transfer (5, 6). Class II enzymes also generate a transient cysteinyl radical but employ adenosyl cobalamin for this purpose (7). Class III enzymes, for which the anaerobic *E. coli* reductase is the prototype, contain an iron–sulfur center and, in their active form, a glycyl radical (8, 9). This radical is stable under anaerobic conditions, but it is oxygen sensitive, and class III enzymes only operate during anaerobiosis. The glycyl radical is generated from *S*-adenosylmethionine (10) by interaction of the reductase with a complex enzyme system present in *E. coli*, consisting of a 17.5-kDa iron protein ("activase") (11), flavodoxin (12), flavodoxin reductase (13), NADPH, and dithiothreitol.

All reductases use their protein radical to generate an activated substrate radical (2, 3), which is subsequently reduced at C-2′. For the reduction, class I and class II enzymes employ enzyme-bound cysteine thiols (2, 7, 14, 15), maintained in the reduced state by transthiolation with thioredoxin or glutaredoxin (16). For the *E. coli* class III reductase, the hydrogen donor has been unknown until now. The extreme oxygen sensitivity of the glycyl radical resulted in the loss of the radical during enzyme purification. This prevented studies of ribonucleotide reduction in the absence of the strongly reducing activating system and made it difficult to identify the reducing equivalents for the reduction of the ribose.

By minor modifications of the previously published purification procedure for the anaerobic reductase, we were now able to prepare an enzyme that maintains a sizeable part of its glycyl radical and reduces CTP in the absence of the activating system. Formate was identified as the hydrogen donor for the reaction. In earlier experiments a fraction named RTF (12) supplied formate required for the reduction of CTP.

EXPERIMENTAL PROCEDURES

Purification of the Anaerobic Reductase. The most important modification of the previously published procedure (17) was stricter adherence to anaerobic conditions. All manipulations were carried out inside an anaerobic box maintained at 4°C. In addition, all solutions including the dATP-Sepharose used for affinity chromatography were purged at length with argon, and manipulations inside the box were done under a stream of argon.

The starting material was *E. coli* JM 109 (DE3) (Promega) transfected with plasmid pREH (11), grown and induced with isopropyl-1-thio-β-D-galactopyranoside as described earlier (17). In a typical experiment, 5.5 g of tightly packed cells was incubated for 40 min with 10 mg of egg white lysozyme in 8 ml of Tris·HCl, pH 7.5/50 mM KCl/1 mM phenylmethylsulfonyl fluoride/0.5% Nonidet P-40. The solution was frozen quickly and thawed three times, followed by centrifugation at 180,000 × *g* for 60 min. The clear supernatant solution (205 mg of protein) was dialyzed overnight against two changes of 200 ml of 30 mM Tris·HCl, pH 7.5/0.5 M KCl/20 mM sodium formate/1 mM phenylmethylsulfonyl fluoride/0.5% Nonidet P-40 (buffer A). The dialysate was diluted with 3 volumes of buffer A and percolated through a 7-ml column of dATP-Sepharose (17) at 1 ml/min. The column was washed with 10 ml of buffer A followed by 5 ml of 30 mM Tris·HCl, pH 7.5/20 mM sodium formate. The anaerobic reductase was then eluted as a sharp protein peak (2.5 ml, 27.5 mg of protein) with 1 mM ATP in the last wash buffer. Aliquots of the solution were stored anaerobically in sealed tubes under liquid nitrogen.

When prepared in this manner, different batches of the anaerobic reductase contained between 2.5 and 3 atoms of iron per protein dimer. EPR spectroscopy demonstrated the presence of up to 0.5 mol of glycyl radical per protein dimer. Specific enzyme activities were between 1300 and 1500 when the enzyme was assayed in the presence of the activating system. In its absence, specific activities usually ranged from 300 to 500. The latter values decreased slowly on storage.

Purification of RTF. Twenty milliliters of the *E. coli* extract was heated at 100°C for 60 min. After centrifugation, 5 M HCl was added to the supernatant solution to give a pH of 1.

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Removal of the precipitate was followed by extraction with several portions of ether. The extract was freed from ether, neutralized with 1 M NaOH, and passed through a 1-ml Sep-Pak C-18 (Waters) column. The flow-through fraction was concentrated to 0.1 ml and constituted RTF. It contained 20 mM formate as determined by an enzymatic assay with formate dehydrogenase (18).

Assays of Enzyme Activity. *Formation of dCTP from CTP.* Incubation conditions for assays in the presence of the activating system were described earlier (10, 11). For assays of the radical-containing enzyme in the absence of the activating system, tubes containing solutions of 1–3 mM [^{3}H]CTP (30 cpm/pmol), 1 mM ATP, 30 mM KCl, 1–5 mM sodium formate, 30 mM Tris·HCl (pH 8.5) in a final volume of 0.05 ml were made anaerobic at room temperature by a 60-min purging with argon on a manifold. The reaction was started by anaerobic addition of the enzyme with a gas-tight microsyringe through the septum of the tubes and stopped after 20 min by addition of 1 ml of 1 M $HClO_4$. The amount of dCTP formed was determined (17). One unit of activity is 1 nmol of dCTP formed per minute. Specific activity is given in units per mg of protein.

Formation of $^{14}CO_2$ from [^{14}C]HCOONa. Assays were done as described above with the radical-containing enzyme using [^{14}C]HCOONa (9800 cpm/nmol). The reaction was stopped with 0.05 ml of 4 M $HClO_4$ added through the septum of the tubes. Argon flushing was continued for 1 hr with the gas leaving the tubes being passed through 2 ml of 2 M KOH. The amount of $^{14}CO_2$ formed was calculated from the radioactivity of the KOH solution.

Formation of ^3H_2O from ^{3}HCOONa. [^{3}H]Formate (8440 cpm/nmol) was used together with [^{3}H]CTP in these assays. Reactions were terminated by exposing the tubes to air. Half of the solution was used for the determination of dCTP. The amount of tritium in water was determined in the other half of the solution by two alternative methods. In early experiments, water was distilled twice in a vacuum into a tube immersed in liquid nitrogen, and the amount of radioactivity was determined in the distillate. A more convenient method proved to be to pass the alkaline solution through a small column of Dowex-1-OH^- and measure the radioactivity of the flow-through fraction. The amount of tritiated water formed, expressed in nanomoles, was obtained by dividing the total radioactivity in the distillate or the flow-through fraction by the specific radioactivity of formate. Both methods gave identical results.

RESULTS

The Activity of RTF Is Due to Its Content of Formate. In earlier work we found that reduction of CTP by the anaerobic reductase required a heat stable, low molecular weight factor present in a boiled extract of *E. coli*, named RTF (12). RTF is not required for the activation of the reductase but serves its function during the actual reduction of CTP. When the activation reaction was monitored by EPR spectroscopy (9), the same amount of glycyl radical was detected whether RTF was present or not (data not shown).

During purification of RTF, several results suggested that RTF was an organic acid. The activity did not bind to a cation-exchange column but bound strongly to anion-exchange columns equilibrated with low-affinity anions such as OH^-. At pH 1, but not at neutral pH, all RTF activity was extracted into ether or chloroform.

During attempts to fractionate RTF with a formate-containing buffer, we recovered enormous amounts of RTF activity, suggesting that formate was responsible for activity. Formate was indeed found to be present in RTF. In a comparison of the effects of formate and RTF on CTP reduction, stimulation by RTF was completely accounted for by its content of formate (Fig. 1). RTF also contained some inhibitory material, which explains the decreased activity at high concentrations of RTF seen in Fig. 1. Treatment of RTF with formate dehydrogenase in the presence of NAD^+ to eliminate formate (18) abolished stimulation of CTP reduction. In a time curve, the loss of stimulatory activity closely followed the decrease in formate concentration (Fig. 2). The slow reaction at the final stages of this experiment is explained by the high K_m (13 mM) of formate dehydrogenase for formate.

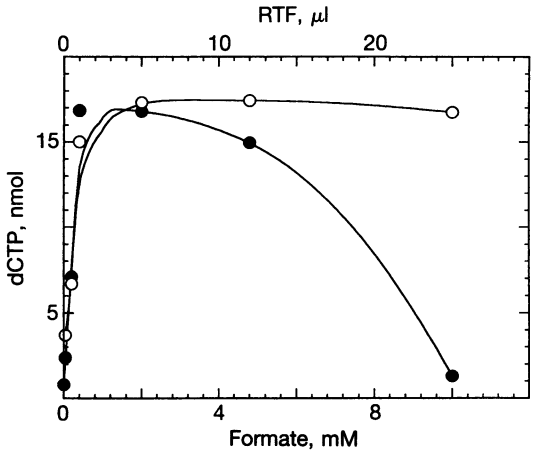


FIG. 1. Formate substitutes for RTF during reduction of CTP. The reductase (2.6 μg) was activated anaerobically as described (10) in the presence of formate (○) or RTF (●) containing 20 mM formate. The reaction was started by addition of [^{3}H]CTP (10), and the amount of dCTP formed during the ensuing 20 min was determined.

These experiments strongly suggest that formate was the only active compound present in RTF. Formaldehyde, formamide, methanol, acetate, bicarbonate, and pyruvate did not replace formate (data not shown). The formate analog hypophosphite strongly inhibited CTP reduction. At 2 mM formate, 50% inhibition occurred at 0.3 mM hypophosphite.

Formate Is Oxidized to CO_2 During CTP Reduction. With the radical-containing enzyme, prepared by the purification method described in *Experimental Procedures*, reduction of CTP only required ATP (as allosteric effector), K^+, Mg^{2+}, and formate. Reduced thioredoxin and glutaredoxin had no effect, and dithiothreitol actually inhibited the reaction (data not shown). It therefore appeared likely that formate functioned as hydrogen donor in the reaction. This was shown to be the case by using ^{14}C- and ^{3}H-labeled formate during the reaction.

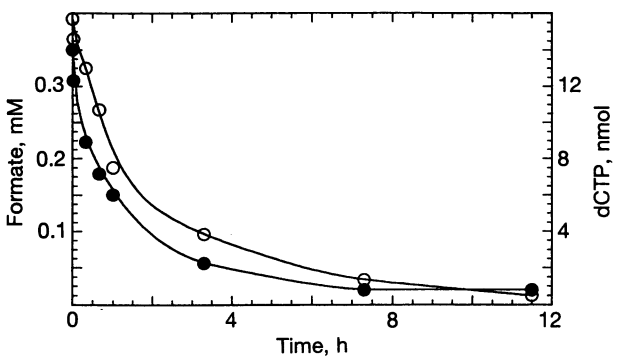


FIG. 2. Formate dehydrogenase abolishes the effect of RTF. Twenty microliters of RTF was diluted in a quartz cuvette to 1 ml with 0.1 M Tris·HCl, pH 8.5/1 mM NAD^+, formate dehydrogenase (Sigma; 1 unit at zero time and 0.5 unit after 1 h) was added, and formate oxidation was monitored from the increase in absorbance at 340 nm (18). At the indicated time intervals, 50-μl portions were removed and heated for 2 min at 100°C, and 25 μl was assayed with 11.5 μg of reductase as described in the legend to Fig. 1. ○, Formate remaining; ●, dCTP formed.

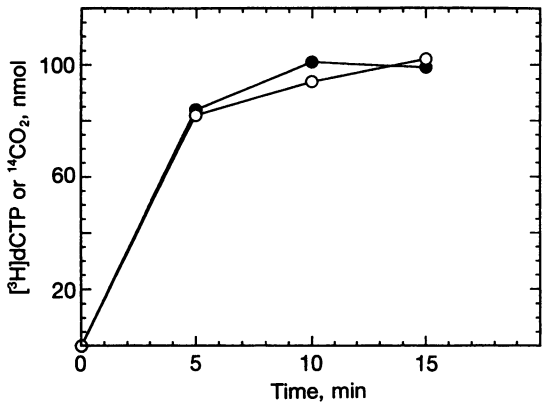


FIG. 3. Stoichiometric formation of dCTP and CO_2. Enzyme (11 μg) was incubated with 2 mM [^{3}H]CTP and 2.5 mM [^{14}C]formate in a final volume of 0.05 ml as described in *Experimental Procedures*, and the amounts of [^{3}H]dCTP (○) and $^{14}CO_2$ (●) formed at different time points were determined.

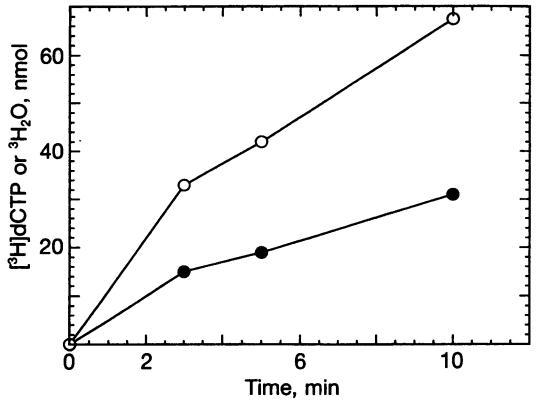


FIG. 4. Tritium is transferred to water from [^{3}H]formate. Enzyme (5 μg) was incubated with 2 mM [^{3}H]CTP and 5 mM [^{3}H]formate as described in *Experimental Procedures*, and the amounts of [^{3}H]dCTP formed (○) and tritium transferred to water (●) at different time points were determined.

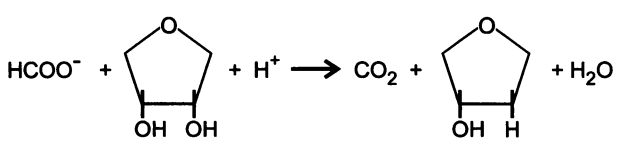
A time curve of the formation of $^{14}CO_2$ from [^{14}C]HCOONa, measured simultaneously with the formation of dCTP from CTP, is shown in Fig. 3. Equimolar amounts of dCTP and CO_2 were formed during the whole course of the experiment. The reaction leveled off after 5 min due to exhaustion of CTP. Clearly, the oxidation of formate ceased once all CTP was reduced.

With [^{3}H]formate, we found in preliminary experiments that no tritium was incorporated into dCTP but, instead, appeared in water. We could thus simultaneously use ^{3}H-labeled CTP and formate to quantify the incorporation of isotope into water in relation to dCTP formation. In a time curve, the two reactions were completely parallel, but there was no 1:1 stoichiometry for dCTP and ^3H_2O formation (Fig. 4). This reflects a selection against tritium during enzyme-catalyzed reduction by a factor of ≈2.3. Accordingly, the specific activity of formate increased with the extent of the reaction. In other experiments in which formate became limiting, the total amount of radioactivity in water at the end of the reaction roughly matched the radioactivity added to the assay as formate (data not shown).

Tritium exchange depended on the presence of all the components required for CTP reduction (Table 1). In the absence of Mg^{2+}, K^+, or CTP almost no isotope was recovered in water. dCTP could not substitute for CTP. Deletion of ATP (a positive effector of CTP reduction) decreased the reaction to 25%, and this value further decreased to 6% if the negative effector dATP substituted for ATP. In other experiments with GTP as substrate, we found that the positive effector dTTP (19) stimulated and the negative effector dATP inhibited the exchange of tritium (data not shown).

DISCUSSION

Class I and class II enzymes use reduced thioredoxin and glutaredoxin as electron donors for the reduction of C-2′ of the ribonucleotide (1, 2, 16). Our results now clearly demonstrate that the anaerobic *E. coli* reductase, a class III enzyme, instead uses formate for this purpose. This enzyme catalyzes the following reaction:



We made this demonstration with an enzyme that during its purification had maintained a good part of its iron–sulfur center and its glycyl radical. Therefore, unlike previous enzyme preparations, this preparation did not require prior reductive activation and showed a much increased specific activity, even though it was not fully active.

Formate is an excellent two-electron reducing agent with a standard oxidation–reduction potential for CO_2/HCO_2 of −420 mV. Formate oxidation to CO_2 is catalyzed by formate dehydrogenases, which can be divided into two separate groups. One group is found in obligate and facultative methylotrophic bacteria as well as in certain strains of yeast and some plants. These enzymes use NAD^+ as electron acceptor and, with few exceptions, require no other cofactors or prosthetic groups (20).

A second large group of formate dehydrogenases, which are commonly found in anaerobic bacteria, utilizes electron acceptors other than NAD^+. These enzymes contain iron–sulfur clusters as well as metals such as molybdenum, tungsten, and selenium. The role of these cofactors in electron transfer is still unclear (21).

The anaerobic ribonucleotide reductase has unique properties as a formate-utilizing enzyme. It contains an iron–sulfur center but no other metals. It is highly specific for ribonucleoside triphosphates as electron acceptors and has a relatively low K_m for formate (0.2 mM). The mechanism of the reaction is unknown, but the participation of the iron–sulfur center during electron transfer from formate to substrate must be considered. Our finding that hydrogen from formate is totally lost to the solvent during catalysis

Table 1. Requirements for the transfer of tritium to water

Components	dCTP, nmol	^3H_2O, nmol
Complete	52	25
− $MgCl_2$	0.8	0.9
− KCl	3.4	2.0
− CTP	—	0.9
− CTP, + dCTP	—	0.4
− ATP	12	6.3
− ATP, + dATP	1.4	1.5
− ATP, − CTP	—	0.3

The complete system contained 2 mM [^{3}H]CTP, 2.5 mM [^{3}H]formate and all the other components detailed for the assay of the radical-containing enzyme (5 μg) in *Experimental Procedures*. Incubation was for 10 min after which time dCTP formation and transfer of tritium to water were determined.

indicates that hydrogen is transferred to a site where it exchanges with the protons of water. Such an exchange occurs with class I and II enzymes and is there attributed to redox-active cysteine residues present in the enzymes as well as in thioredoxin and glutaredoxin (22, 23). Also in the present case the exchange might occur on redox-active thiols of the reductase. The primary isotope effect is about 2.3, suggesting that "hydride" transfer in the active complex is rate-limiting. This effect is in the range of the isotope effects reported for several formate dehydrogenases (20).

We have earlier speculated that ribonucleotide reduction was required for the evolution of DNA (1). Since DNA appeared before oxygen became abundant in the atmosphere, a primitive ribonucleotide reductase would have operated under anaerobic conditions. Among the known reductases, the class III *E. coli* reductase appears to be the closest possible relative of such an "ur" reductase. The structure and requirements of this enzyme appear more primitive than those of class I and II enzymes. In line with this, we now find that formate replaces the more complicated redoxin systems as a reductant. This finding also links class III reductases still closer to pyruvate formate lyase, another important and probably primitive enzyme involved in anaerobic metabolism (24). The many common features of the two enzymes make us speculate that both arose from a common ancestor during evolution.

What about the evolution of class I and II enzymes? Once photosynthesis started, ribonucleotide reduction required an enzyme that could function in the presence of oxygen. The similarities of the highly complicated allosteric mechanisms regulating the substrate specificity of all reductases may suggest that the new enzymes evolved from a primitive class III enzyme (1, 9), in spite of large differences in primary and quaternary structures. However, the solution of this question awaits further crystallographic work, which will allow a comparison of the tertiary structures of class II and III enzymes with that of the R1 protein of class I reductases (6).

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