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CONTINUAL PRESENCE OF OXYGEN AND IRON REQUIRED FOR MAMMALIAN RIBONUCLEOTIDE REDUCTION: POSSIBLE REGULATION MECHANISM

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A radical-free preparation of a highly purified ribonucleotide reductase from calf thymus was shown to generate an M2-specific tyrosine free radical on incubation with iron and dithiothreitol in the presence of air. The radical is essential for activity but once formed has a half-life of about 10 min. Using the calf thymus enzyme, there is a continual requirement of oxygen and iron for ribonucleotide reduction indicating a continual regeneration of the radical during enzyme catalysis . We therefore propose that one way a cell may regulate ribonucleotide reductase activity is by controlling the generation of M2-specific tyrosine free radicals within existing M2 molecules.

Mammalian ribonucleotide reductase is composed of two non-identical subunits, proteins M1 and M2 (1-3). Mouse fibroblast cells selected for resistance to hydroxyurea showed a 30-fold increase in M2 activity but normal amounts of M1 (4). EPR studies of such cells revealed the presence of an M2-specific tyrosine free radical structure first identified and shown to be essential for activity in the E. coli ribonucleotide reductase (5,6). The hydroxyurea resistant cells showed at least a 20-fold increase in M2 tyrosine free radical content but only about a 3-fold increase in the amount of M2 protein (4,6). This suggested that cells may have a mechanism for controlling ribonucleotide reductase activity by modulating the fraction of radical-containing, active M2 within a pool of preexisting, radical-free, inactive M2 molecules (4).

A highly purified ribonucleotide reductase from calf thymus consisting essentially of protein M1 and M2 in the approximate ratio 19:1 by weight was used to study the complex allosteric regulation of the enzyme and also as a source for further purification leading to homogeneous M1 and M2 (2,3,7). No M2-specific tyrosine free radical was however detected in this preparation or

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in a homogeneous calf thymus protein M2 (3). We now show that such a radical can be induced in the highly purified calf thymus ribonucleotide reductase by incubation with a dithiol, iron and oxygen. The radical formed is labile and needs to be continually regenerated during enzyme catalysis as seen by a continual requirement for oxygen and iron. Our observations are compatible with a role of M2 radical regeneration in the cellular regulation of ribonucleotide reductase activity.

MATERIALS AND METHODS. Highly purified ribonucleotide reductase from calf thymus was material after affinity chromatography on dATP-Sepharose with a specific activity of 15-20 U/mg and consisting of about 95% protein M1 and 5% protein M2 (1). E. coli thioredoxin reductase and thioredoxin were gifts of Dr. A. Holmgren, Department of Chemistry I, Karolinska Institutet.
Enzyme assay. Ribonucleotide reductase activity was determined by measuring the reduction of [^{3}H] CDP (1). The CDP reduction mixture contained 50 mM Tris-Cl, pH 7.6, 0.1 M KCl, 8 mM N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid buffer, pH 7.6, 3 mM ATP, 6 mM $MgCl_2$, 10 μM $FeCl_2$, 10 mM dithiothreitol and 0.5 mM[^{3}H]CDP with a specific activity of 20000cpm/nmole.
EPR measurements. EPR first derivative spectra were recorded at 77 K on a Bruker ER 100 Laboratory EPR instrument equipped with an ER 040 XR microwave bridge and a cold finger Dewar containing liquid nitrogen. Determinations of free radical concentrations in the samples were performed by comparing double integrals of spectra with that of a frozen solution of 1 mM $CuCl_2$, 10 mM EDTA.

Results and Discussion

Tyrosine free radical of highly purified calf thymus ribonucleotide reductase. Highly purified ribonucleotide reductase preparations from calf thymus, consisting of proteins M1 and M2 in the ratio 19:1 by weight, showed specific activities of 20-30 U/mg but still contained less than 0.008 nmole of M2-specific tyrosine free radical/mg as measured by EPR spectroscopy (limit of detection). This should be compared to partially purified enzyme preparations from hydroxyurea-resistant mouse fibroblast cells which, in the presence of an excess of added pure M1 subunit, showed similar specific activities but contained 0.14 nmol of tyrosine radical/mg of protein (4,6,8). In such partially purified preparations the radical could be destroyed by hydroxyurea or 1-formylisoquinoline thiosemicarbazone treatment and then regenerated under assay conditions or simply by incubation in dithiothreitol, iron and oxygen (6,8).

To study possible radical regeneration in a radical-free, highly purified calf thymus ribonucleotide reductase preparation, an enzyme solution was incu-

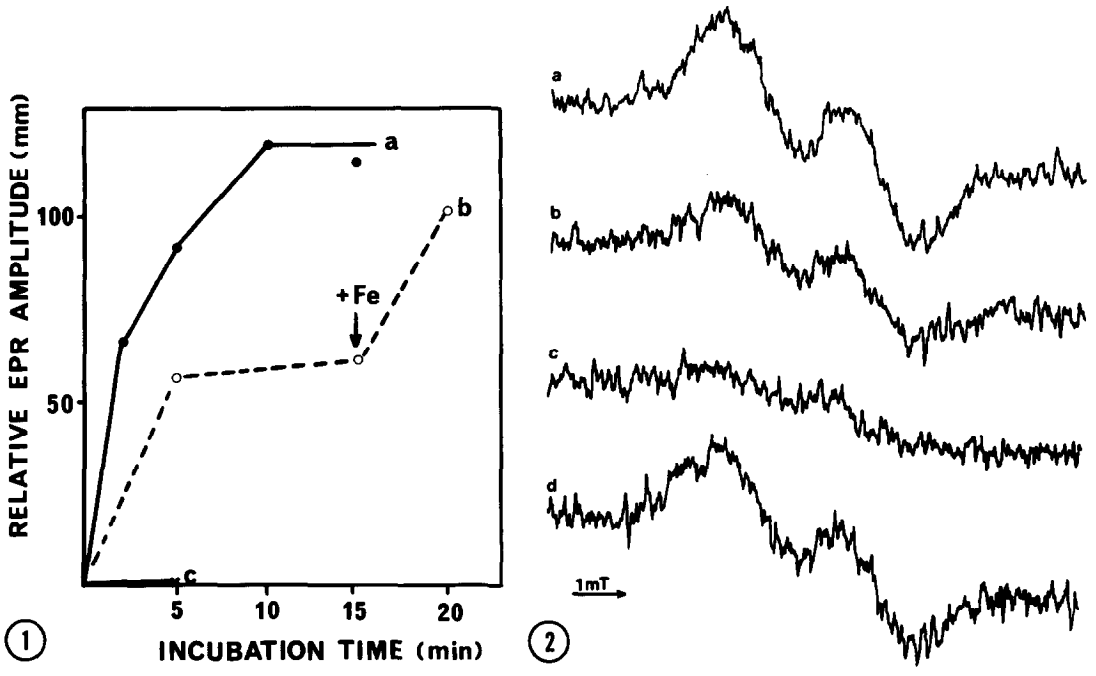


Fig. 1. Regeneration of the tyrosine free radical in the highly purified ribonucleotide reductase from calf thymus. To remove free iron, the enzyme solution was first passed through a Sephadex G-25 column (sample to column volume 1:10) washed with 10 mM EDTA followed by Chelex-treated 50 mM Tris-Cl, pH 7.6, 0.1 M KCl. Into a series of EPR tubes, 180 µl of the Sephadex eluate having a protein concentration of 3.8 mg/ml were pipetted. To one series of tubes, 5.4 µl of a solution containing 0.33 M dithiothreitol and 0.33 mM $FeCl_3$ were added followed by incubation at 37° (curve a) or at 0° (curve c). At the indicated time points, samples were frozen in liquid nitrogen and their EPR spectra recorded. To another series of tubes, 3.6 µl of a Chelex-treated solution of 0.5 M dithiothreitol were added followed by incubation at 37° (curve b). After recording the EPR spectrum, the 15 min sample was thawed, 2 µl of a solution of 1 mM $FeCl_3$ were added and the incubation continued for 5 min at 37° (curve d). In the EPR measurements, the microwave power was 91.5 milliwatts and the modulation 0.63 millitesla.

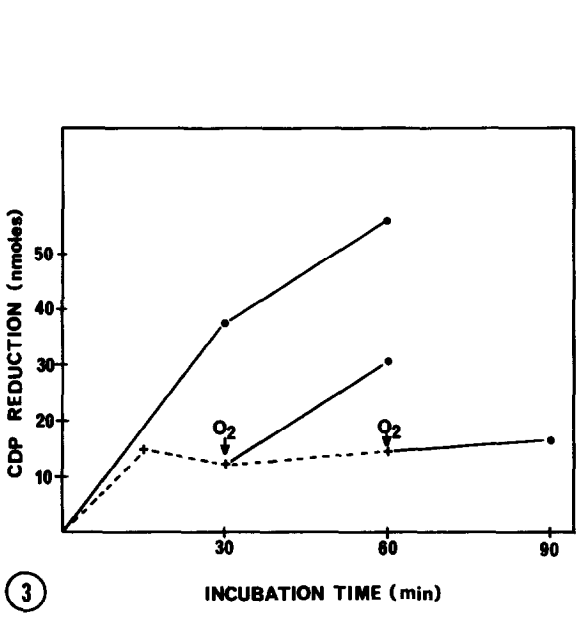
Fig. 2. Stability of the calf thymus protein M2 tyrosine free radical. A solution of a highly purified calf thymus ribonucleotide reductase (1.0 mg of protein) was added to a Thunberg-type modified EPR tube containing 10 µmoles Tris-Cl, pH 7.6, 20 µmoles KCl, 1.8 nmoles $FeCl_3$ and 1.8 µmoles dithiothreitol in a final volume of 205 µl. After 10 min incubation at 37° in the presence of air, the tube was made anaerobic by first exposing the solution to a flow of oxygen-free argon for about 30 min at 0° and then by repeated evacuation and flushing with the same gas. For further details see text. In the EPR measurements the microwave power was 91.5 milliwatts and the modulation 0.63 millitesla (mT).

bated as described in the legend to Fig. 1 . An M2-specific tyrosine free radical EPR signal was indeed obtained on incubation in the presence of dithiothreitol and iron with a maximal value of 0.09 nmol radical/mg of protein after 10 min at 37° (Fig.1). Omission of iron resulted in a slower and less

pronounced radical regeneration which however was markedly stimulated also when the iron was added 15 min after the dithiothreitol. No radical signal was observed on incubation at 0° or under anaerobic conditions (data not shown). This induction of a tyrosine free radical in the calf thymus enzyme very much resembles radical regeneration in the iron-free apo-B2 of the E. coli ribonucleotide reductase (9).

Stability of the tyrosine free radical structure of calf thymus protein M2. A solution of ribonucleotide reductase was preincubated with iron-dithiothreitol in the presence of air to regenerate the tyrosine free radical. After making the solution anaerobic, it was frozen in liquid nitrogen and the EPR spectrum was recorded (Fig. 2a). A value of 0.07 nmol radical/mg of protein was obtained. This value decreased to 0.04 nmol/mg after thawing the sample and incubating for 10 min at 37° under anaerobic conditions (Fig. 2b). Thawing followed by another 10 min incubation at 37° under anaerobic conditions resulted in a complete disappearance of the radical signal (Fig. 2c). Finally, the sample was thawed, air was bubbled through the solution, and the sample incubated for 10 min at 37° under aerobic conditions. This treatment almost completely regenerated the tyrosine free radical (Fig. 2d). From these data we conclude that in the presence of iron-dithiothreitol under anaerobic conditions when continual regeneration of the radical cannot occur due to lack of oxygen, the radical is destroyed with a half life of about 10 min.

Continual presence of oxygen required for ribonucleotide reduction by the highly purified calf thymus reductase. To investigate if continual reactivation of the M2 radical was necessary also during normal assay conditions, a time curve of CDP reduction under anaerobic conditions was performed (Fig. 3). First the enzyme solution was preincubated with iron-dithiothreitol in the presence of air to regenerate the M2 radical. Without this preincubation no CDP reduction was obtained under anaerobic conditions (data not shown). The dithiothreitol could be replaced by 0.6 mM NADPH, 0.7 μM thioredoxin reductase and 5 μM thioredoxin but in one single experiment the activity with the thioredoxin system was about half of the activity obtained with dithiothreitol. In



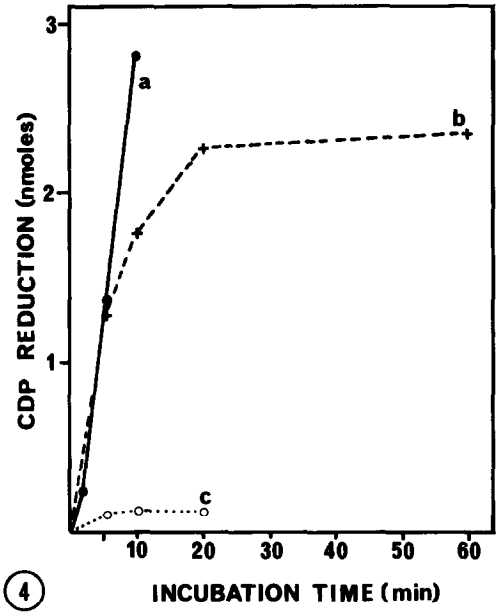


Fig. 3. Ribonucleotide reduction under aerobic and anaerobic conditions. A highly purified ribonucleotide reductase preparation (118 µg of protein) was added to four Thunberg tubes containing 500 µl of a CDP reduction mixture minus CDP and the solutions were preincubated in the presence of air for 5 min at 37°. The tubes were made anaerobic by repeated evacuation and flushing with oxygen-free argon for 5 min and then [^{3}H]CDP was added anaerobically from a side arm. Air was immediately added to one tube serving as an aerobic control and then all tubes were incubated at 37°. At the indicated time points, samples were removed for dCDP determinations. After 30 and 60 min of anaerobic incubation, air was admitted to two separate tubes after that samples had been removed for dCDP analyses, and incubation was continued for 30 min. Aerobic conditions •—• , anaerobic conditions +--+ .

Fig. 4. Ribonucleotide reduction in the absence and presence of EDTA. a) A solution of a highly purified calf thymus ribonucleotide reductase (102 µg of protein) was added to a complete CDP reduction mixture at 0° to give a final volume of 250 µl. The mixture was then incubated at 37° and aliquots of 50 µl were withdrawn at the indicated time points to determine the amounts of dCDP formed. b) The same incubation mixture as in a) but this time without [^{3}H]CDP was preincubated for 5 min at 37°. Then 2.5 µl of a solution of 0.2 M EDTA were added followed by the [^{3}H]CDP and the incubation was performed as described above. c) The same as in a) but 2.5 µl of a solution containing 0.2 M EDTA were added to the incubation mixture before the addition of substrate and shifting from 0° to 37°.

the aerobic control CDP reduction was approximately linear with time for 60 min (Fig. 3). In contrast, under anaerobic conditions, the reduction stopped already after 15 min of incubation. Addition of air to the assay mixture after 30 min of anaerobic incubation immediately restored CDP reduction to values close to the aerobic control. On the other hand, addition of air after 60 min of anaerobic incubation had very little effect on CDP reduction. The results show that a continual presence of oxygen is needed to maintain the active,

radical-containing form of protein M2 necessary for catalysis. This is quite different from the E. coli ribonucleotide reductase where anaerobiosis during the assay of the homogeneous enzyme has no effect on catalysis (M. Sahlin and B.-M. Sjöberg, personal communication).

Ribonucleotide reduction under limiting iron conditions resembles the reduction under limiting oxygen conditions. EDTA is a very potent inhibitor of the highly purified calf thymus ribonucleotide reductase when present during the assay (50% inhibition at 10 μM EDTA in the absence of added iron). However, EDTA has very little effect on the M2-specific free radical when added to a radical-containing enzyme preparation from hydroxyurea-resistant mouse fibroblast cells (data not shown). This effect is quite different from that of hydroxyurea or 1-formylisoquinoline thiosemicarbazone, which both specifically destroy the already present tyrosine free radical of mammalian ribonucleotide reductase (6,8). To test if the strong EDTA inhibition observed during the assay might be due to inhibition of radical regeneration, we made a time curve study of CDP reduction catalyzed by the calf thymus enzyme in the presence and in the absence of EDTA (Fig. 4). When the enzyme was preincubated in iron-dithiothreitol to regenerate the tyrosine free radical before the addition of EDTA followed by substrate to start the reaction, the rate of CDP reduction was the same as in a control without EDTA for about 5 min (Fig. 4 a and b). Then the reduction in the presence of EDTA slowed down and finally stopped after about 20 min of incubation. If, on the other hand, EDTA was added to the enzyme solution prior to the addition of iron-dithiothreitol, very little CDP reduction was observed on the addition of substrate (Fig. 4c). Separate EPR measurements confirmed that in the presence of 2 mM EDTA no tyrosine radical signal could be detected after incubation of the enzyme for 10 min at 37° in iron-dithiothreitol (cf. Fig. 1). We conclude from these experiments that EDTA interferes with the regeneration of the tyrosine free radical, most likely by removing iron necessary for the reaction. Once the radical-iron center is formed, however, it seems to be stable to EDTA and the inhibition observed follows the same pattern as the one seen under anaerobic conditions. It is inte-

resting that both under limiting oxygen and iron conditions, enzyme catalysis stops after 15 - 20 min incubation indicating that oxygen and iron are required for the same process, i.e. the reactivation of the tyrosine free radical. The observed lability of the tyrosine radical in calf thymus ribonucleotide reductase supports the hypothesis that one way of controlling this enzyme may be by regulating the generation of active, radical-containing M2 molecules from preexisting, radical-free ones (4). Each of the components necessary for radical regeneration, iron, oxygen and a dithiol, may be limiting in this reaction. Further studies will show if radical regeneration is involved in the control of ribonucleotide reductase activity during the cell cycle (cf. 10).

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