Ribonucleoside diphosphate reductase is a functional and structural component of the replication hyperstructure in *Escherichia coli*

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ABSTRACT

INTRODUCTION

Escherichia coli has a single chromosome of 4639 kbp that replicates once every cell cycle. The time required for the elongation of chromosome replication, C period, may vary with growth rates longer than one hour and with a number of mutations affecting the replication process (1). As this period of time, contrastingly to what is normal in eukaryotes, is not related with cell cycle length, τ period, bacterial cells regulates the requirement of having two full chromosomes to be segregated before cell division by two comprised procedures: by initiating replication once every cell cycle, therefore once every τ minutes, and by dividing C + D minutes after initiation takes place, or D minutes after the end of replication (2).

DNA replication initiates in *oriC*, a 248 bp sequence located at minute 84.57 of the genetic map and nucleotide 3 923 360 (3). All proteins required for the completion of the replication process have to be fulfilled at this initiation step. Once replication initiates neither RNA nor protein synthesis is further required, replication is fully replicated in the absence of synthesis of any these two macromolecules. This means that all proteins required for the whole process must be present at this initiation step. It is estimated that at least 23 proteins for initiation and more than 25 for elongation, in different copy number, are required (4, 5). If to the strict replication mechanism the requirement for precursor biosynthesis are included, ten to fifteen proteins must be further added. All these proteins must furthermore function coordinately and corregulated which makes evident that they must be associated in a complex extremely well regulated. Cells are not a bag of enzymes (6) but a network of colocalized proteins that were called “metabolons” or “protein machines” (7) which confer to sharing enzymes unique catalitic properties (8). Replication machine must be one of the most complex of these machines, not only due to the number

of partakers but to the regulation among most of them and to the sensing of other cellular process, such as the DNA structure and membrane fluidity.

A higher level in the concept of these multienzymatic complexes are "hiperstructures" propossed by Norris and col. (9, 10, 11). These hiperstructures include lipid bilayers, DNA, proteins and related metabolons that assemble to achieve a particular function and disassemble when no longer required. These hiperstructures could be responsible for the asymmetrical structure of the bacterial cell and compartmentation of metabolites. One of the proposed hiperstructure is SeqA hiperstructure involved in the sequestration of newly replicated *oriC* and in the assembly and maintenance of the replication complex during elongation (11, 12).

Two kind of enzyme machines have been proposed for DNA replication, the replication complex, also termed "replisome" and "replitase", containing helicases, polymerases, primase and enzymes directly related with DNA synthesis, and the dNTP-synthesizing complex which includes enzymes related with precursors biosynthesis (13, 14, 15). In vitro studies with purified proteins from *E. coli* have shown the specific joining of DNA polymerase III Holoenzyme with DNA through β subunit and with helicase DnaB and Primase through τ subunit making a complex of 17 proteins (16, 17, 4, 18, 19, 20). The dNTP-synthesizing complex has received a number of evidences from studies with viruses to mammalian cells (reviewed in 14).

An important and not well understood problem with DNA replication in all living cells is the extreme limitation of the precursors pool. *E. coli* replicates its chromosome by two forks in about 50 minutes in cells with a doubling time lower than 100 minutes at 37ºC (1). This time means that each replication fork synthesizes DNA at a rate of 1500 n s^{-1}. At the same time, RNA polymerase is transcribing DNA at an average rate of 45 n s^{-1}. In contrast to this difference in polymerization, the dNTP pool is about ten times smaller that the NTP pool (21). This discrepancy was observed early by Werner (22) who asked how the intracellular concentration of dNTP could be sufficient to support the observed rate of DNA replication. Werner found that the rate of labeling of DNA by exogenous labeled thymine or thymidine reaches its maximum value long before the TTP pool is fully labeled. This could indicate that TTP is synthesized at the same time and place where replication takes place and it is not accumulated at the cell pool, which point out to the comparmentation of precursors. Besides this difference in pools, dNTPs are highly

specialized molecules, as they have few roles outside DNA replication, and this functionality is highly localized at only a few intracellular sites.

In a work on the isolation of a DNA replication system bound to membrane in rat liver and hepatomes, Baril and col. demonstrated the incorporation of ^{3}H-thymidine into DNA in their *in vitro* system (23). They found the association of DNA polymerase and at least three enzymes participating in the production of dNTP precursors, one of them being the NDP reductase. From this work they proposed the presence of a multi-enzyme replication complex in eukaryotes that would contain key enzymes for the production of dNTP, as well as the enzymes and factors participating directly in DNA replication (23).

A noteworthy work with permeabilized chinese hamster embryo fibroblast cells showed that NDP are used for DNA synthesis more efficiently than dNTP, and incorporation of exogenous dNTP only occurs when NDP reductase is abolished by hydroxyurea (24). These results made them to propose a complex of DNA precursor synthesizing enzymes juxtaposed with the replication complex which were termed the "replitase".

This idea of a multi-enzyme complex synthesizing DNA precursors and associated to the replication machinery in eukaryotes was extended by Mathews and coworkers to prokaryotes. These authors determined *in vitro* aggregation and cross-linking of proteins related with the biosynthesis of dNTP by sedimentation analysis in T4 infected *E. coli* cells to find eight phage-coded enzymes and two enzymes of host origin associated in a 1.5 mDa complex (25, 26, 27, 28, 29, 30).

A more interesting semi-*in vivo* study with plasmolyzed bacterial cells showed *i*) that dNMP's are incorporated into DNA more efficiently than dNTP's, *ii*) that TTP nucleotides are degraded before they are incorporated into DNA, and *iii*) that cyclamidomicin, a specific inhibitor of nucleoside disphosphate kinase of *E. coli*, inhibits the incorporation of both dNMP and dNTP, suggesting that precursors for DNA synthesis must pass through diphosphates intermediates (31).

These works gave rise to the idea of the channeling in the synthesis of precursors of DNA synthesis, and to the compartmentation and concentration of dNTP in a pouch associated with the replication machinery (13, 32, 14, 33, 34, 24, 15). By comparing between the maximal DNA synthesis activity and the TTP concentration *in vivo* and *in*

vitro, Mathews and Sinha suggested that dNTP should be 3- to 4-fold concentrated at this replication pouch to give maximal rate of replication (33).

Although this association between dNTP-synthesizing complex and the replication complex has been proposed by several groups, no direct evidence has been obtained for this association. We believe that our recent work establishing the relationship between the NDP reductase, a specific protein of the dNTP-synthesizing machinery, and protein Tus, a specific protein of the replication apparatus, is the first evidence of this association to give a replication multi-complex machinery or hyperstructure (35).

NDP reductase

Ribonucleoside diphosphate reductase, NDP reductase, of *E. coli*, prototype of class I reductases, common for most prokaryotes and eukaryotes from viruses to man, is the only specific enzyme required for enzymatic formation of deoxyribonucleotides, the precursors for DNA synthesis. The active enzyme is a 1:1 complex of two nonidentical subunits, called proteins B1 and B2, each consisting of two nearly identical polypeptide chains, coded by genes *nrdA* and *nrdB* respectively (36, 37), and contains a free tyrosyl radical stabilized by an iron center (for review see 38, 39, 40).

In NDP reductase, a single catalytic center directs the reduction of four different substrates, ADP, GDP, CDP and UDP to their correspondent deoxyribonucleotides. DNA replication requires a balanced supply of four dNTP which explains for the complex allosteric control of the enzyme. The large B1 subunit is endowed with specific allosteric sites that regulate both general enzymatic activity and specificity (38, 41).

Although about 3000 nucleotides have to be consumed per second when bacteria replicates its chromosome with two replication forks, very small pool of dNTP are accumulated into the cells. This pool would permit replication for not longer than half minute (22). To circumvent this shortage canalization of their biosynthesis and compartmentation of precursors have been proposed (32, 14, 33, 34, 31, 15). This proposal suggests that dNTP are produced by a synthesizing complex that would concentrate the final products in a small compartmentation precisely where they are used by replication machinery. This compartmentation will increase precursors concentration to the required amount to obtain maximal DNA polimerization, but total cell concentration will be maintained low. This high concentration but in a low amount means that along DNA

replication precursors have to be synthesized continuously and therefore NDP reductase should be keeped in constant activity to maintain a constant balanced mini-pool of precursors. The alteration of this balanced synthesis might explain the origin of mutations by misincorporation during replication (42).

To satisfy this changing demand of four deosynucleotides, NDP reductase must be tightly associated to the replication machinery. The above mentioned work of Mathews and coworkers obtained evidences of association of this enzyme to others related with precursors biosynthesis and were named dNTP-synthesizing complex (13, 14, 15). In our lab we have found evidences for a direct association of this enzyme, and therefore the synthesizing complex, with the replication machinery, that has been partially published (35).

RESULTS AND DISCUSSION

Hydroxyurea inhibits replication and causes cell death

To show that NDP reductase is required all along the replication elongation, hydroxyurea (Hu), a specific inhibitor of this enzyme (43, 44), was added to a exponentially growing culture of *E. coli* strain JK607 (*thyA arg his thi malA* λ^P *rpsL mtl xyl su*) and acid precipitable ^{3}H-thymidine incorporation was measured. None radiactive precursor incorporation was detected after adding the drug, revealing that pool of precursors is not enough for a detectable synthesis, as addressed above.

Together with DNA synthesis inhibition, Hu causes cell death. This loss of cell viability strictly depends on the number of active replication forks. When the number of replication forks were increased by decreasing thymidine concentration, sensibility to Hu increased correspondingly (Fig. 1). On the other hand, when new replication cycles were inhibited by amino acid starvation or by adding rifampicin, no cell death by Hu was observed after all replication forks reached the terminus (data not shown). If after two hours of starvation, when all replication cycles had been concluded, amino acid were added together with Hu, no cell death was observed. These results clearly suggest that the lethal effect of Hu must be due to the damage produced to the DNA structure by the stalled replication forks. Not initiated replication forks or forks just about to leave the initiation step seem to be resistant to this damage.

Inhibition of DNA synthesis by Hu is fully reversible

When Hu was removed from a bacterial culture treated up to three hours, replication restarted instantaneously. To know if NDP reductase was reversible and all stalled replication forks were equally reversible, we inhibited enzyme activity with Hu and new initiations by rifampicin, and after different periods of time only Hu was removed to detect whether present replication forks could continue functional elongation until the terminus. Functionallity of restarted forks was detected by measuring runout replication and flow citometry. As figure 2 illustrates, runout replication shows that all preexisting forks were fully functional after removal of the drug without requiring new protein synthesis. Flow cytometry corroborates this conclusion by showing full replicated chromosomes (Fig. 3.d).

When Hu was added without inhibiting new initiations, removal of the drug after two hours of treatment together with addition of rifampicin to stop new initiations gave a relative residual DNA synthesis of about 170% (Fig. 4) that gives mostly 4 and 8 full replicated chromosomes (Fig. 3.f). This high DNA synthesis implies that during Hu treatment new replication forks are being organized at the initiation step and that they have surpassed the RNA polymerase requiring step. After removal of Hu, all stalled replication forks restarted elongation until termination, those forks stalled in the midle of the elongation step and those initiated during the Hu treatment. From both techniques we can measure the proportion of present origins that initiate a new cycle of bidirectional replication (45). Measurements in several bacterial strains show this reinitiation fraction to occurs in 99.6±11.5 per cent of present origins. These results mean that Hu inhibits NDP reductase activity without interfering with any other functionality of the replication hyperstructure, that the initiation of replication is not affected by this drug and that during the treatment time initiation potential is being accumulated that will run into elongation as soon as the drug was removed.

Mutation *nrdA101* does not behaves as an elongation mutation

The only know mutation in genes coding for NDP reductase is mutation *nrdA101*, originally named *dnaF101* (45) and recently named *nrdA1* (*E. coli* Genetic Stock Center: cgsc.biology.yale.edu/cgsc.html). This mutant allele codes for a thermosensitive B1 subunit which makes a NDP reductase that loses 99 per cent of its activity in 2 minutes at 42ºC *in vitro* (37). Consequently, as pool of dNTP is extremely low and its continuous activity is strictly required for the elongation of replication (see above), incubation of this mutant at the restrictive temperature should inhibit elongation instantly as Hu does, however replication remains for more than 40 min in strain JS1018 (Fig. 1 in ref. 35). Marker frequency analysis and flow cytometry show that these elongations do not end at the terminus of replication but they stop stochastically throughout the chromosome (Fig. 2 and 3 in ref. 35). These results implies that NDP reductase must have a termoresistance period protected by some subcellular structure. This enzyme has been proposed to be part of a complex for the biosynthesis of dNTP (14), therefore the association with this complex might explain for this effect.

Incubation of this mutant NDP reductase at 30ºC *in vitro* gives an activity 3.7 per cent of a wild type enzyme (37), consequently, growing the mutant strain at the permissive temperature makes the elongation of replication to last about 160 min. A direct consequence of this low activity is the lowering of precursors and the lengthening of the C period but, if replication machine acquired its precursors from the cellular pools, this deffect should be arranged in a simple way by increasing expression of *nrdAB* genes to obtain the required pool (47, 48, 49). The lengthening of the elongation of replication implicates that this regulation does not remediates the shortage of precursors wich leads to the idea that a constant number of enzyme molecules are present per biosynthesis complex and per replication machinery and this can not be increased.

Mutation *nrdA101* affects chromosome segregation and cell division

The growth of strain JS1018 at the restrictive temperature causes inhibition of cell division and filamentation (Table 1, Fig. 5.d). Other *nrdA* conditional mutants have also showed a similar phenotype (50, 51). This effect have being attributed to the inhibition of replication under conditions that RNA and protein synthesis continues which could tentativelly suggest that the inhibition of replication without at the same time inhibiting cell mass synthesis will inhibit cell division and cause filamentation. But the inhibition of replication by inhibiting NDP reductase activity by adding Hu at the permisive temperature affects cell size only to a small extent. Cell division is very slightly affected by inhibiting replication with the drug since Hu treatment up to 3 hours gives normal cell size but it increases when the cell division inhibitor cefalexin is present (Table 1, Fig. 5.b and 5.c). Consequently filamentation of the *nrdA101* mutant at the non permissive temperature is not a direct consequence of inhibiting replication but an additional effect of destroying the replication hyperstructure upon cell division. Furthermore, when restrictive temperature treatment was carried out together with rifampicin addition, no filamentation (Table 1, Fig. 5.e) and no cell death were observed. These observations are explained by the fact that at the non permissive temperature in the presence of rifampicin all replication forks keep their functionallity and complete replication but no new replication cycle is initiated, therefore none active replication hyperstructure is affected by this treatment.

Fluorescence microscopy of DAPI stained cells shows that the inhibition of NDP reductase by Hu does not affect the DNA content. On the other hand, incubation of the

thermosensitive mutant to the restrictive temperature strongly affects the DNA content and distribution along the cells, fluorescence is very irregularly distributed along the cells and an elevated proportion of short anucleated cells appears (Table 1). These results add an additional argument to the idea that the effects of restrictive temperature in the *nrdA101* mutant are not simply due to the inhibition of the NDP reductase activity but to a greater effect upon the replication hyperstructure. The effects upon nucleoid positioning and segregation bring us to suggest that replication hyperstructure is also connected with the chromosome segregation machinery and its alteration by the temperature will affect both process.

Table 1. Cell size and number of anucleated cells in strain JS1018 growing in M9 minimal medium after different treatments for 3 hours.

treatment	cell size (μm)	anucleates (%)
expon. grow	3.4±1.0	0.5
Hu	4.6±1.1	1.0
Hu + cephalexin	8.8±2.3	<0.1
42ºC	17.1±14.6	35
42ºC (anucle. cells)	2.3±1.4	
42ºC + rifampicin	3.3±0.8	<0.1

It has been suggested that the replication hyperstructure (9) or the replication factory (53, 52) should be implicated in the chromosome positioning and segregation in *E. coli*. An alteration of this macrostructure not only will affect replication but also chromosome movements along the cell cycle. The above described alterations of both replication and movements of chromosomes show the severe effects of the thermosensitive *nrdA101* mutation, far from inhibiting solely NDP reductase activity, and its consequent structural implication in the replication hyperstructure.

Other conditional DNA replication mutants, *dnaB* (54), *dnaG* (*parB*) (55), *dnaX*, *gyrA* (*parD*), *gyrB* (*parA*) (56), *parCE* (57), accumulate DNA-less cells under

nonpermissive growth conditions thought to be due because they undergo cell division without nucleoid segregation. In the case of *dnaG* it has been suggested that DnaG form part of the replisoma and its attachment to the membrane would be required for the segregation machinery (55). We propose that primase or NDP reductase might not be the specific link for nucleoid segregation but a structural part of the replication machinery and the functional organization of this hyperstructure should be required not only for replication but also for chromosome segregation (58). This proposal confers the replication hyperstructure the functionallity of a mobil centromere.

Thermal inactivation of the replication fork requires protein synthesis

It could be thought that the arrest of replication forks at the restrictive temperature in mutant *nrdA101* could result from a simple inactivation of NDP reductase activity, notwithstanding the observed refractory time described above. If this were correct, there should be no effect of RNA or protein synthesis inhibition at the high temperature. When an exponentially growing culture of JS1018 was shifted from 30º to 42ºC together with the addition of rifampicin or chloramphenicol, all replication forks were functional for the complete replication cycle, accumulating the same amount of residual DNA synthesis as the treatment with the drugs at 30ºC (Fig. 4 from ref. 35). In the absence of RNA or protein synthesis, all replication forks continued replication until they ended at the chromosome terminus, giving fully replicated chromosomes as shown by marker frequency and flow cytometry (Fig. 2D and 3D from ref. 35), cell size did not change (Fig. 5.e) and not anucleated cells were formed at the restrictive temperature. These results mean that, in the absence of protein synthesis, mutant NDP reductase is fully functional for the complete replication period, of 154 min in this strain, confirm the model of protection of the NDP reductase by a hyperstructure, and support the idea that replication arrest does not result from inactivation of the mutant enzyme by the restrictive temperature. We suggest that the behavior of the thermosensitive mutant at the non permissive temperature could be caused by a destabilizing protein, a protein repair protein, the synthesis of which seems to be induced by the appearing of a thermally altered protein. In the absence of this protein, mutant NDP reductase and replication hyperstructure are resistant to thermal inactivation during the complete replication time. Furthermore, in the absence of protein synthesis the restrictive temperature does not affect nucleoid positioning and segregation which suggest

that the alteration of these functionalities are due to the modification of the replication hyperstructure.

Protein Tus inactivates replication forks under non permissive conditions in strain *nrdA101*

With the aim of finding the protein required in the arrest of the replication forks, we tested for the implication of Tus. This protein is known to arrest replication forks by its specific interaction with a *ter* sequence by antagonizing the action of DnaB helicase required for the progression of the elongation (59). The *E. coli* chromosome contains six *ter* sites located in the terminus region, consisting in a sequence of 22 nucleotides (60, 61). Other partially homologous sequences may exist throughout the chromosome that could diminish fork movement and facilitate disorganization of any altered replication hyperstructure, or the stalled replication hyperstructure be less stringently assembled which makes the NDP reductase to loss its thermal protection. This disorganization could explain by itself the arrest of fork movement at 42ºC in a *nrdA101* mutant strain.

To test this idea, we carried out similar experiments with strain JQ434 which has the allele *nrdA101* and is devoid of Tus protein. The shift in the incubation temperature of an exponentially growing culture of JQ434 from 30ºC to 42ºC gave a residual DNA synthesis similar to that obtained after the addition of rifampicin at 30ºC (Fig. 5 from ref. 35). Both the kinetics of the synthesis and the final relative accumulation of DNA at 42ºC indicate that the absence of Tus permits a much longer thermostability of NDP reductase and a longer processivity of replication forks than when it is present. Thus, Tus protein can explain the shorter stability of the replication fork in the strain JS1018, and seems to have a role in destabilizing the partially altered replication complex, resulting in the inhibition of elongation of replication in the *nrdA*101 mutant at the restrictive temperature.

The kinetics and the final accumulation of DNA synthesis after the shift of a culture of JQ434 from 30° to 42°C could be explained as due to a runout replication after the inhibition of new initiations. If this were correct, full replicated chromosomes should be detected by marker frequency and flow cytometry analysis. However, after 4 h at 42°C, these two methods show the same results, a random inhibition of elongation and very different from the inhibition of new initiations by rifampicin (Fig. 6 and Fig. 7 from ref.

35). This shows that, in the absence of Tus, incubation of the *nrdA*101 mutant at the restrictive temperature permits a much longer processivity of the replication forks, but instead of stopping at the *ter* sites they end stochastically throughout the chromosome. When rifampicin was added at the time of the temperature shift, a similar residual DNA synthesis was found than without the drug but all chromosomes forks ended at the replication terminus giving fully replicated chromosomes. These results show that, in the absence of Tus, mutant NDP reductase and replication forks are functional for longer than a C period at the restrictive temperature.

Occasional replication arrests are frequent in *E. coli* and a source of chromosome instability (62, 63, 64) and our results indicate that Tus could contribute to cause these arrests or could cause the halt of replication forks by recognition of somewhat similar *ter* sequences. Results point out that this arrest could partially destabilize the joining of replication proteins at the hyperstructure and make mutant NDP reductase not protected and sensitive to the high temperature. During that time hyperstructure might be vulnerable to repair proteins that can detect modified proteins and substitute them by new ones. In the *nrdA101* mutant at the high temperature NDP reductase could not be repaired because no new wild protein could be synthesized. The absence of Tus would permit these replication hyperstructures to be functional for a much longer period of time.

This rationalization confers a new functionality to protein Tus during replication but demonstrate that it is not the protein whose synthesis is required to arrest replication forks at the restrictive temperature in the *nrdA101* mutant.

Concluding remarks

The hereinabove presented work let us attain the following conclusions and suggest the following rationalization.

1. NDP reductase coded by *nrdA101* allele is inactivated in a few minutes by the temperature *in vitro*, but *in vivo* it is physically protected from its thermal inactivation by its assotiation with other components of the replication hyperstructure.

2. Given this protection, NDP reductase keeps its functionality for longer than 150 min, the C period of strain JS1018. This long period means that once inserted in the replication hyperstructure NrdA101 behaves as a non mutant product.

3. The arrest of replication forks, probably due to Tus activity along the chromosome, could cause replication hyperstructure to become transiently unstable. In this position a thermosensitive NDP reductase would somewhat lose its protection and become structurally partially affected.

4. The alteration of this protein, or the effect of its alteration upon the replication hyperstructure, could induce the synthesis of a protein that could launch protein repair process to restore the affected complex. At the non permissive temperature this restauration could not be completed as any new free NDP reductase will be denatured by the temperature.

5. In the absence of protein synthesis, the former repair protein could not be synthesized and the partially affectd NDP reductase could come again protected by the hyperstructure at the end of its arrest, and the replication fork will restart replication until the terminus.

6. Hu inhibits NDP reductase outside and inside replication hyperstructure and stops DNA synthesis all at once. This inhibitory activity is fully reversible and after removal all replication forks recover normal activity. When this treatment is performed in the presence of rifampicin, residual replication informs that all replication forks are reactivated after removal of Hu and repair or substitution of the enzyme is required.

7. During the time replication forks are suspended by Hu, cell mass still increases and new initiation potential is being accumulated beyond the rifampicin sensitive step. The number of *oriC* initiated and ready for elongation can be measured by adding rifampicin about ten minutes before removing Hu and quantifying residual DNA synthesis. In this way 100 per cent of *oriC* were detected to reinitiate using different bacterial strains.

8. Hu causes cell death. This lose of viability is strictly proportional to the number of replication forks which shows that they are the target of the drug actiion.

9. Inhibition of DNA replication by the non permissive condition in the *nrdA101* mutant strain inhibits cell division and causes filamentation.

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