Allosteric Regulation of *Trypanosoma brucei* Ribonucleotide Reductase Studied *in Vitro* and *in Vivo*

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*Trypanosoma brucei* is the causative agent for African sleeping sickness. We have made *in vitro* and *in vivo* studies on the allosteric regulation of the trypanosome ribonucleotide reductase, a key enzyme in the production of dNTPs needed for DNA synthesis. Results with the isolated recombinant trypanosome ribonucleotide reductase showed that dATP specifically directs pyrimidine ribonucleotide reduction instead of being a general negative effector as in other related ribonucleotide reductases, whereas dTTP and dGTP directed GDP and ADP reduction, respectively. Pool measurements of NTPs, NTDPs, and dNTDPs in the cultured bloodstream form of trypanosomes exposed to deoxyribonucleosides or inhibited by hydroxyurea confirmed our *in vitro* allosteric regulation model of ribonucleotide reductase. Interestingly, the trypanosomes had extremely low CDP and CTP pools, whereas the dCTP pool was comparable with that of other dNTPs. The trypanosome ribonucleotide reductase seems adapted to this situation by having a high affinity for the CDP/UDP-specific effector dATP and a high catalytic efficiency, $K_{cat}/K_m$, for CDP reduction. Thymidine and deoxyadenosine were readily taken up and phosphorylated to dTTP and dATP, respectively, the latter in a nonsaturating manner. This uncontrolled uptake of deoxyadenosine strongly inhibited trypanosomal proliferation, a valuable observation in the search for new trypanocidal nucleoside analogues.

$\text{Trypanosoma brucei}$ is an African unicellular eukaryote that lives extracellularly in the mammalian bloodstream and central nervous system as well as in the guts and salivary glands of tsetse flies. Residing in its mammalian host, it causes a fatal disease called sleeping sickness. There is an urgent need to find new chemotherapy against this disease because the current ones are limited by toxicity as well as an increasing resistance among the trypanosomes (1).

Ribonucleotide reductase (2) is a key enzyme in DNA synthesis because it catalyzes the reduction of ribonucleotides to deoxyribonucleotides, a reaction assisted by a protein-bound or a 5'-deoxyadenosyl covalamin-derived free radical. Primarily based on the nature of this radical, the ribonucleotide reductases are divided into three classes where most of the eukaryotic and some of the prokaryotic ones belong to class I. The enzymes in this class are heterodimers formed from two large (R1) and two small (R2) polypeptides. The R1 protein binds substrates and allosteric effectors, whereas the R2 protein contains a tyrosyl radical essential for enzyme activity. The radical can be scavenged by the one-electron reductant hydroxyurea, a drug that has been in clinical use against leukemias (3).

Except those from the Herpesviridae family (4), all ribonucleotide reductases studied are allosterically regulated. The nonviral class I enzymes have two types of allosteric effector binding sites called the activity site (lacking in certain bacterial ribonucleotide reductases specifically called class Ib) and the specificity site (2). The activity site determines the overall activity of the enzyme by binding either ATP (positive effector) or dATP (negative effector), whereas the specificity site determines which will be reduced of the four substrates. With dATP (at such a low concentration that it does not bind to the activity site) (5, 6) or ATP bound to the specificity sites, CDP and UDP are reduced, whereas dTTP and dGTP gives GDP and ADP reduction, respectively. X-ray diffraction of R1 protein crystals containing substrates and effectors (7) has revealed that the effectors control specificity by mediating small conformational changes of the protein rather than being in direct contact with the substrate. Binding of effectors also greatly stimulate R1/R2 protein interaction, explaining the low activity observed in the absence of effectors (8).

Although ribonucleotide reductase has a central role in keeping the deoxyribonucleotide levels balanced inside the cell, it is not the only system. Because CDP and UDP require the same effectors for their reduction, ribonucleotide reductase cannot balance the level of dCTP versus dTTP. In mammalian cells only a small fraction of the dTTP in a cell comes from UDP reduction as explained by the low $V_{max}/K_m$ of ribonucleotide reductase for UDP compared with the other substrates (9). Instead, the majority of the dTTP formed has gone through dCMP deamination. The enzyme that performs this job, dCMP deaminase, is activated by dCTP and inhibited by dTTP (10). A third level of regulation can also be found in the equilibrium with the extracellular medium between uptake and excretion of deoxyribonucleosides. Deoxyribonucleoside kinases and 5'-nucleotidases (11), the former ones often allosterically regulated, directs the primary control of these processes. It is not known whether $T. brucei$ contains a dCMP deaminase, and the only information available about its deoxyribonucleoside kinases is a thymidine kinase that has been partially purified and characterized (12). Similar to the mammalian thymidine kinase, the one from $T. brucei$ is inhibited by dTTP. Recently, an expressed sequence tag from $T. brucei$ identified as a thymidine kinase was released in the GenBank data base (accession number AA098695).

We have earlier cloned, expressed, and characterized the ribonucleotide reductase from $T. brucei$ (13) and studied its CDP reduction. A major difference compared with the mammalian ribonucleotide reductase was that dATP did not inhibit...
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CDP reduction. Now, we have made a thorough investigation of how the allosteric effectors influence the \( K_m \) and \( V_{max} \) for all the four ribonucleoside diphosphate substrates, a study that confirmed that dATP does not inhibit enzyme activity but instead is the primary positive effector for CDP and UDP reduction. The experiments made on the enzyme level were complemented with studies on the NTP and dNTP pools in trypanosomes grown in culture. We found that the deoxyribonucleotide metabolism of \( T. brucei \) in many aspects is unique, not only in the allosteric regulation of ribonucleotide reductase but also in the properties of the deoxyribonucleoside kinases. The finding of an uncontrollable salvage of deoxyadenosine should be helpful for the development of nucleoside analogue therapies against sleeping sickness.

Another unexpected finding from our studies of the NDP, NTP, and dNTP pools in \( T. brucei \) was that the CDP and CTP levels were extremely low, a situation that is not reflected on the dCTP pool. How the \( T. brucei \) ribonucleotide reductase copes with the low CDP substrate pool will be discussed.

**Experimental Procedures**

**Determination of NTP and dNTP Pools by HPLC**—The bloodstream form of \( T. brucei \) (strain 221) (kindly supplied by Fred Oppendoes, ICP, Trop 7439, avenue Hippocrate 74, B-1200, Brussels, Belgium) was maintained at 37 °C and 7.2% CO\(_2\) in Hirumi’s modified Iscoves Medium (HMI)-9 medium (14) lacking thymidine and Serum Plus but containing 10% fetal calf serum. The omission of thymidine did not affect the growth of the parasites. Trypanosomes (200 ml), harvested in late logarithmic phase, were chilled on ice for 5 min before being collected at 3,000 \( \times g \) for 5 min at 4 °C. Subsequently, the pellet was resuspended in 1 ml of culture medium, transferred on an Eppendorf tube, and collected at 20,000 \( \times g \) for 1 min at 4 °C. The NTP, NDP, and dNTP pools were not affected by the time on ice (varied between 5 and 15 min), the centrifugation time (varied between 5 and 15 min), the centrifugation speed (varied between 2,000 and 20,000 \( \times g \)) or how many washings in culture medium that were made (varied between 1 and 3 times). After the medium wash, the collected trypanosomes were disintegrated by pipetting them up and down in 500 ml of ice-cold 0.6 M trichloroacetic acid containing 15 mM MgCl\(_2\). One nmol of unlabeled dGTP was added at this step to monitor the losses during the preparation. The resultant solution was centrifuged at 20,000 \( \times g \) for 1 min at 4 °C, and the supernatant was extracted twice with 1.1 \( \times \) the volume of Freon (78% v/v)/2-propanol (22% v/v) (15). One-tenth of the sample was evaporated to dryness in a Speedvac (Savant) and dissolved in 100 \( \mu l \) of water. This fraction was used for quantification of ribonucleoside triphosphates by a 4.6 \( \times \) 125-mm Partisphere SAX-5 HPLC column (Whatman) and run isocratically in 0.5 M ammonium phosphate, pH 3.4, and 2.5% (v/v) acetonitrile at 1.5 ml/min. The nucleotides were quantified by measuring the peak heights and comparing them to a standard curve.

The remaining 90% of the sample was pH-adjusted with 25 \( \mu l \) of 1 M NaHCO\(_3\), pH 8.9, and the deoxyribonucleotides were separated from the ribonucleotides by boronate affinity chromatography (Affigel-601, Bio-Rad) as described by Shewach (16). The flow-through (2 ml), containing deoxyribonucleotides, was adjusted to pH 3.4 by approximately 20 \( \mu l \) of 3 M HCl, vortexed for 30 s to get rid of the evolving CO\(_2\), and finally separated by HPLC (the same conditions as described for the nucleoside triphosphates). The acidified sample (maximally 1 ml could finally be separated by HPLC (the same conditions as described for the nucleoside triphosphates). The resultant solution was centrifuged at 20,000 \( \times g \) for 1 min at 20 °C. When used in combination with dATP and dGTP, the ammonium sulfate pellet was resuspended in a volume of 50 mM Hepes, pH 7.3 (this concentration and pH was used throughout the protocol), sufficient to give a conductivity of 80 millisiemens/cm or lower (typically 100 ml, if starting from bacteria grown in 6-liter of Terrific Broth). The protein solution was applied to a dATP-Sepharose column (4 ml) that subsequently was washed with 20 ml of 50 mM Hepes, pH 7.3, 20 ml of 0.5 mM ADP + Hepes, 20 ml of Hepes, 20 ml of 0.2 mM KC1 + Hepes, 20 ml of Hepes, and 20 ml of 1 mM ATP + Hepes. An elution rate of 0.5 ml/min was used throughout all steps. Finally, the R1 protein was eluted with 10 mM ATP + Hepes using the same pump speed as the washing steps. The first two-thirds of the eluate was precipitated and dissolved in a Sephadex G-50 column with 10% glycerol, 0.1 mM KC1, and 50 mM Tris-HCl, pH 7.6. The inclusion of 10% glycerol increased the stability of the protein during freezing and thawing. However, because it was not completely stable, the solution was aliquoted and never frozen and thawed more than once.

**Filter Binding Assay to Measure Nucleotide Binding to the R1 Protein**—\( ^{8}-\)HdGTP was purchased from Amersham Pharmacia Biotech, diluted in unlabeled dGTP to a specific activity of 230 cpmp/ml, and stored as a neutral solution at −20 °C. Protein R1 (10 \( \mu g \)) was incubated for 5 min in 50 mM Tris-HCl, pH 7.6, 50 mM KC1, 10 mM DTT, 6.4 \( \mu M \) MgCl\(_2\), 4.5 \( \mu M \) tritiated dGTP, and varying amounts of competing cold nucleotides. In those cases where the total nucleotide level exceeded 3 mM, the MgCl\(_2\) concentration was increased to constitute twice the level of nucleotides. The amount of bound and free nucleotide was determined by scintillation counting of aliquots of the solution before and after centrifugation through a membrane (17).

**Assay of Ribonucleotide Reductase Activity**—Enzyme, substrates, and effectors were incubated at 37 °C for 30 min in a buffer described earlier (18). The concentrations of effectors used were 1.6 mM ATP, 1.6 (M Tris-HCl, pH 7.6, 500 \( \mu M \) dGTP, 1.6 \( \mu M \) ATP, and 20 \( \mu M \) dATP. The amount of R2 was always 1.5 \( \mu M \), whereas the concentration of R1 was chosen so that not more than 10% of the substrate was converted during the assay so that the R2 protein was in at least 5 times molar excess over the R1 protein. The substrate used were \( [5^{-\text{H}}] \)dCIP (Amersham Pharmacia Biotech), \( [2^{-\text{C}}] \)UDP (kindly supplied by Peter Reichard, Karolinska Institute, S-171 77 Stockholm, Sweden), \( [8^{-\text{C}}] \)GDP (kindly supplied by Lise Bagger, Bio-chemicals), and \( [3^{-\text{H}}] \)GTP (kindly supplied by Fred Oppendoes, ICP, Sweden). They were diluted with unlabeled nucleotides from Sigma into specific activities of 37,000, 3,320, 3,570, and 1,400 cpnmol, respectively. All substrates except CDP were purified by DEAE-Sephadex as described earlier (19). Subsequent to the enzymatic reaction, the assay mixture was boiled for 5 min and centrifuged, and the protein pellet was discarded. A 1 ml solution of NH\(_4\)HCO\(_3\), pH 8.9, was added to the supernatant to a final concentration of 50 mM, and the products were separated from the substrates by boronate affinity chromatography as described for the dNTP pool determination. One ml of the flow-through (totally 2 ml) was mixed with 50 \( \mu l \) of 3 M HCl and 10 ml of Instagel Plus (Packard) before counting. The cpm of a blank containing all assay ingredients except enzyme was subtracted from all values. With CDP as the substrate, we originally used an alternative protocol (18) involving Dowex-50 chro- matography to separate products from substrates. However, the low sensitivity of this method combined with the low \( K_m \) for CDP with ATP and dATP as effectors made us choose the boronate affinity chromatography when these effectors were used. The results from all assays were plotted as enzyme activity versus substrate concentration (taken on both sides of the \( K_m \) value), and \( V_{max} \) and \( K_m \) were determined by fitting the data to a hyperbola by the PRISM version 2.0 software (GraphPad Software, Inc.).

**Drugs**—Hydroxyurea (Sigma) was dissolved in water and stored as a 0.5 mM solution at −20 °C. Tetrahydouridine (Calbiochem) was dissolved in water, flushed with argon gas, sealed under airtight conditions, and stored as a 20 mM solution at −20 °C. When used in combination with deoxycytidine, cytidine, or cytosine, the trypanosomes were preincu-
bated for 30 min with 40 μM tetrahydrouridine before the addition of nucleoside/base. Coformycin (Calbiochem) was stored as a 3 mM aqueous solution at −20 °C.

RESULTS

Determination of Nucleotide Pools in T. brucei—Initially we followed an extraction protocol proposed by Khym (15) and optimized by Van Haverbeke and Brown (20). In that scheme, the cells are disintegrated by ice-cold 10% trichloroacetic acid, the debris was discarded, and the nucleotide containing solution was extracted with a Freon-trioctylamine mix to remove the acid. Like Van Haverbeke and Brown, we observed significant losses of nucleotides during the Freon-trioctylamine step (up to 40% of the dITP, which we used as an internal control in the trypanosome extract). However, inclusion of 15 mM MgCl₂ in the trichloroacetic acid reduced these losses effectively (99% recovery of dITP). Similar improvements were obtained if standard nucleotide solutions in 10% trichloroacetic acid were used instead of trypanosome extracts. We also observed that without MgCl₂, the losses affected dITP and dGTP much more than dATP, dTTP, and dCTP.

Extracted nucleotides were separated directly by HPLC for quantification of NTPs or run through a boronate affinity column before the HPLC step for quantification of dNTPs. The boronate matrix absorbs the ribonucleotides under alkaline conditions, pH 8.9. Because the ribonucleotides, with the exception of CTP, have slightly different retention times than the deoxynucleotides when run on HPLC, we could observe that 99.9% of the UTP, ATP, and GTP in the trypanosome extracts were removed during this step. As judged from standard nucleotide solutions, CTP is as efficiently removed during this step as the other NTPs.

NTP, dNTP, and NDP Pools in T. brucei—We decided to relate the dNTP pools to the total nucleoside triphosphate pool (determined to be 2300 ± 530 nmol/10⁹ cells) rather than the amount of cells or DNA content because we found less variation in the results using this parameter. However, we routinely checked the amount of cells to ensure that the conditions of exposure did not alter the total nucleoside triphosphate pool.

An average of 19 experiments showed the relative levels of nucleotides to be 1.2 ± 0.4% CTP, 12 ± 2% UTP, 68 ± 1% ATP, 16 ± 2% GTP, 0.41 ± 0.09% dCTP, 0.85 ± 0.2% dATP, 0.44 ± 0.07% dTTP, and 0.15 ± 0.03% dGTP (Fig. 1, A–B). Given that the mean volume of 10⁹ trypanosomes has been reported to be 58 μl (21), we could calculate that the average intracellular concentrations of the nucleotides are 26 μM CTP, 280 μM UTP, 1600 μM ATP, 380 μM GTP, 9.4 μM dCTP, 19 μM dATP, 10 μM dTTP, and 3.5 μM dGTP. We were immediately struck by the extremely low CTP content in the trypanosomes. The relative CTP level was nearly 10 times lower than in the mouse fibroblasts, Balb/3T3, which we used as control (Fig. 1A). On top of that, the CTP level was further reduced to only 30% of its original value when the trypanosomes were grown into stationary phase and harvested 24 h later.

Because ribonucleotide reductase uses ribonucleoside diphosphates as substrates, we wanted to measure them as well. Crucial for these experiments is that the much larger NTP pools are not degraded to NDPs. We ensured that no degradation of NTPs occurred during the harvesting of trypanosomes by checking if varying the length and conditions of each individual step had any influence on the NDP and NTP/dNTP pools (for details see “Experimental Procedures”). We also checked with standard NDP/NTP solutions that no degradation occurs during the trichloroacetic acid treatment and beyond. The result from the NDP quantification is shown in Fig. 1C. Similar calculations as for the NTP and dNTP pools give average intracellular concentrations of 4 μM CDP, 23 μM UDP, 270 μM ADP, and 120 μM GDP. The pyrimidine NDP pools, particularly the CDP pool, are very low, a situation that is not reflected in the pyrimidine dNTP pools, which are not lower than the purine ones (Fig. 1B). The CDP pool is in fact less than half of the dCTP pool, indicating the high efficiency of ribonucleotide reductase for CDP reduction.

Purification of the Trypanosome R1 Subunit—We discovered that the purified R1 protein from before (13) gave two closely spaced bands when run on a 7.5% polyacrylamide gel (14-cm separation gel). The two bands were transferred to a polyvinylidene difluoride membrane and individually sequenced by the N-terminal sequence of the R1 protein, whereas the upper band was identified as ClpB. This is a chaperone protein in E. coli that has a protein-stimulated ATPase activity (22). Because it has been reported that the ATPase activity is inhibited by ADP that most probably binds to the same site as ATP, the affinity chromatography of the R1 protein on dATP-Sepharose was supplemented with a 0.5 mM ADP washing step. This improvement reduced the ClpB content to virtually undetectable levels, leaving a homogenous R1 protein. Subsequently, the R1 protein was kept in a buffer containing 10% glycerol to compensate for the loss of stability toward freezing and thawing that followed the removal of ClpB.

Filter Binding Studies of the R1 Protein—Previously (13), we have shown that the R1 protein has two different binding sites for dATP. We could repeat this result with the pure R1 protein as well (data not shown). We also wanted to investigate the binding of ATP and dCTP to the specificity sites because it was not made in our earlier work (13). ATP, dCTP, and dATP (as a comparison) were allowed to compete with tritiated dGTP for binding to the R1 protein. As seen in Fig. 2A, the binding of ATP and dCTP is very low, with ATP needed in 5,000 times and dCTP in 10 times higher concentration than dATP to have a corresponding effect on dGTP binding.

The Allosteric Regulation of the Recombinant Trypanosome Ribonucleotide Reductase—In our previous paper (13), we con-
included that the specificity and activity nucleotide effector sites together determine the substrate affinity of the *T. brucei* ribonucleotide reductase. Because the activity site can be in 3 different states (empty, dATP-bound, or ATP-bound) and the specificity site in 6 states (empty or binding ATP, dATP, dGTP, dTTP, or dCTP), 18 different combinations of effectors can be imagined. We have simplified this complex situation by selecting those combinations we considered physiologically most important, namely 1.6 mM ATP (the physiological concentration), 1.6 mM ATP + 0.5 mM dGTP, 1.6 mM ATP + 0.5 mM dTTP, and 0.1 mM dATP. At these concentrations ATP saturates the activity sites, dGTP and dTTP saturates the specificity sites, and dATP saturates both sites as judged from our previous nucleotide binding results (13). The rational behind making the dGTP and dTTP experiments in the presence of ATP was that we wanted to have the activity sites at physiological status while we determined the function of the specificity sites. We tested dATP alone because it can bind both types of allosteric sites and therefore would give a situation hard to interpret if combined with ATP.

Previously (13), we determined the allosteric regulation of CDP reduction using a constant substrate concentration of 0.5 mM. At this high concentration, the amount of substrate is close to saturating levels, and therefore, our experiments predominantly showed how the effectors control V_max. Because the allosteric effectors may influence *K_m* as well, we found it necessary to expand these results using varied substrate concentrations. In Fig. 2B, we have plotted the enzyme activity versus substrate concentration. At high substrate levels, the regulation is, as expected, very similar to what we described previously with dTTP as the optimal effector. However, at low substrate concentration dATP and ATP are considerably better effectors than dTTP. The data used for Fig. 2B were fitted to a hyperbola by nonlinear regression and *K_m* and *V_max* was calculated. These results as well as the corresponding ones from UDP, GDP, and ADP reduction are presented in Table I. As seen in this table, the *K_m* for CDP with dATP or ATP as effector is more than a magnitude less than with dTTP. The values from Table I were used to construct Fig. 2C, where we have plotted how the catalytic efficiency, *K_m*/*V_max*, is influenced by the effectors. We can conclude that CDP reduction is directed by dATP (and to some extent ATP), UDP reduction is directed by dATP, GDP reduction is directed by dTTP, and ADP reduction is directed by dGTP.

Finally, we checked the role of the activity site effectors (Fig. 2D) and dCTP on the trypanosome ribonucleotide reductase activity. The substrates were used at the *K_m* concentration, the specificity site effectors were used at concentrations saturating enzyme activity, and [dATP] was at 50 μM, where it readily binds the activity sites but does not compete with the specificity

TABLE I

| Substrate | Effectora | *K_m* | *V_max* |
|-----------|-----------|-------|---------|
| CDP       | ATP       | 3.2 ± 0.55 | 1.1 ± 0.33 |
| dATP      | 4.0 ± 0.58 | 26 ± 1.0 |
| dGTP/ATP  | 91 ± 9.4  | 14 ± 0.5 |
| dTTP/ATP  | 140 ± 9   | 66 ± 2.0 |
| UDP       | ATP       | 72 ± 15   | 18 ± 1.4 |
| dATP      | 9.6 ± 1.4 | 25 ± 0.9 |
| dGTP/ATP  | 91 ± 3.5  | 76 ± 0.09 |
| dTTP/ATP  | 62 ± 11   | 5.8 ± 0.5 |
| GDP       | ATP       | 500 ± 84  | 18 ± 1.4 |
| dATP      | 410 ± 10  | 13 ± 0.15 |
| dGTP/ATP  | 840 ± 67  | 4.6 ± 0.23 |
| dTTP/ATP  | 10 ± 1.7  | 32 ± 0.9 |
| ADP       | ATP       | 480 ± 97  | 20 ± 1.7 |
| dATP      | 860 ± 270 | 45 ± 7.8 |
| dGTP/ATP  | 33 ± 5.5  | 50 ± 3.3 |
| dTTP/ATP  | 260 ± 32  | 52 ± 2.5 |

*a* The concentrations of the effectors were 1.6 mM ATP, 100 μM dATP, 500 μM dTTP + 1.6 mM ATP, and 500 μM dGTP + 1.6 mM ATP.

*b* nmol of substrate converted/min and mg of R1 protein.
Fig. 3. A, inhibition of *T.* brucei ribonucleotide reductase by hydroxyurea. CDP (0.5 mM) was used as substrate, and dATP (100 μM) was used as effector. The R1 protein has been used at 2.5 times molar excess over the R2 protein. The enzyme activity is expressed as nmol of substrate converted/min and mg of R2 protein. B, the dependence of trypanosome proliferation on hydroxyurea concentration without hydroxyurea (■), 0.01 mM hydroxyurea (▲), 0.06 mM hydroxyurea (●), 0.08 mM hydroxyurea (squad), 0.1 mM hydroxyurea (triolo), 1 mM hydroxyurea (□), and 20 mM hydroxyurea (○). Each curve is an average of two experiments. C and D, the effect of hydroxyurea on the trypanosome dTTP (●), dATP (■), dCTP (▲), and dGTP (△) pools. C, the cells were resuspended in fresh medium before the exposure to hydroxyurea. The *abscissa* shows the time of 20 mM hydroxyurea exposure. Note that the scale is broken. D, trypanosomes treated by 20 mM hydroxyurea for 14 h were released in a hydroxyurea-free medium. The total NTP pool denotes CTP + UTP + ATP + GTP + dCTP + dTTP + dATP + dGTP.

site effectors. As seen in Fig. 2D, ATP stimulated the dTTP-mediated GDP reduction and inhibited the dGTP-mediated ADP reduction. Similarly, dATP stimulated the enzyme activity when combined with dTTP, but it did not inhibit dGTP-mediated ADP reduction. ATP (1.6 mM) + dCTP (2 mM) was not the optimal effector combination for any substrate and as compared with the optimal effectors in Fig. 2C, the activity was 38% for UDP, 43% for GDP, and 13% for ADP reduction. In our previous paper (13), we showed that dCTP did not stimulate any detectable CDP reduction.

A possible source of error might be that some of the effector ATP is degraded to ADP. The formed ADP can then compete with the substrate studied. To resolve this potential source of error, we measured the amount of ADP in a typical assay mixture (including enzyme and effectors but without substrate) by an HPLC run under the same conditions as described for the dNTP pool measurements. From this experiment we could conclude that the assay mixture supplied with 1.6 mM effector ATP contained 24 μM ADP (1.5% of ATP), an amount that did not change during the incubation. Because the concentration of ADP is lower than the *K*ₐ for ADP under all conditions tested, it should have a minor influence on our experiments.

**Effects of Hydroxyurea on Trypanosome Ribonucleotide Reductase and Proliferation**—We wanted to determine the sensitivity of the trypanosome ribonucleotide reductase both *in vivo* and *in vitro* to hydroxyurea. First, the enzyme was incubated under assay conditions with increasing concentrations of hydroxyurea (Fig. 3A), resulting in increased inhibition of enzyme activity. Hydroxyurea also blocked parasite proliferation with an IC₅₀ of approximately 80 μM (Fig. 3B), fully comparable with the IC₅₀ of 60 μM that has been reported for mammalian cells (23). The inhibition of 1 mM hydroxyurea on trypanosome proliferation was fully reversible at 2 h. However, the ability of the trypanosomes to recover proliferation was gradually lost with time (data not shown).

**Effects of Hydroxyurea on the dNTP Pools**—Trypanosomes were harvested and resuspended in fresh medium containing 20 mM hydroxyurea. As seen in Fig. 3C, this led to a rapid decrease of all the deoxyribonucleotide pools during the first 5 min of exposure. As soon as dGTP, which is the lowest pool, is used up, the rapid decrease in the other pools ceases, possibly because of the resulting block in DNA synthesis.

We also checked if the inhibition of dNTP synthesis imposed by hydroxyurea was reversible by releasing the hydroxyurea-treated (14 h) trypanosomes into fresh medium. As seen in Fig. 3D, all dNTP pools start to increase, although not simultaneously. The dGTP pool is already fully recovered after 5 min, followed by dATP (30 min) and finally, the pyrimidine dNTP pools (120 min). The order of recovery is compatible with our *in vitro* data that dTTP (the major dNTP at the onset of hydroxyurea release) turns on the reduction of GDP, dGTP turns on the reduction of ADP, and dATP turns on the reduction of CDP and UDP. The 120-min levels of the dNTPs in Fig. 3D differ from the ones in Fig. 1B, an observation that possibly can be explained by an S-phase synchronization of the hydroxyurea-released cells.

**Effect of Deoxyribonucleosides on the dNTP Pools**—We wanted to further test the *in vivo* regulation of ribonucleotide reductase by feeding trypanosomes deoxyribonucleosides and observing how the increase of a particular dNTP pool influences the others. Neither deoxyxycytidine (1 mM) nor deoxyguanosine (1 mM) seemed to be taken up by the trypanosomes because we could not detect any change in the dNTP pools. The inclusion of 40 μM tetrahydrouridine, which inhibits the cytidine deaminase in the serum, did not change the results with deoxycytidine. In contrast to deoxycytidine and deoxyguanosine, 0.5 mM thymidine was readily converted to dTTP, which increased during the first hour of treatment and then stabilized (data not shown). In Fig. 4A, we show that thymidine salvage is saturated at high concentrations, indicating an allosterically regulated thymidine kinase. The increase in the dTTP pool is accompanied by an increase in the dGTP and decrease in the dCTP and dATP pools, supporting our *in vitro* data that dTTP directs GDP reduction. We also measured if trypanosome proliferation is blocked by the presence of thymidine in the medium and found that they are essentially insensitive to thymidine concentrations as high as 10 mM (data not shown).

Like thymidine, the trypanosomes could very efficiently take up deoxyadenosine and phosphorylate it to the triphosphate (Fig. 4B). However, no saturation was observed, and dATP increased to levels where it even surpassed the level of ATP. The soaring dATP pool was accompanied by an equally sized decrease in ATP, which was evident also when the nucleotide levels were related to the amounts of cells instead of the total amount of nucleoside triphosphates. In Fig. 4C we show that
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Fig. 4. Influence of deoxyribonucleosides on dNTP pools and trypanosome proliferation. A, the pools of dTTP (△), dATP (●), dCTP (■), and dGTP pools (▽) were measured after a 1-h exposure to the different thymidine concentrations. Note that the scale of the ordinate changes after the breakpoint. The total NTP pool denotes CTP + UTP + GTP + dCTP + dTTP + dATP + dGTP. B, the pools of ATP (○) and dATP (◇) were measured after a 1-h exposure to the different deoxyadenosine concentrations in the presence of 5 μM EHNA (open symbols) or without drug (filled symbols). C, effect of 0.5 mM deoxyadenosine on the trypanosome ATP (○), dATP (●), dTTP (■), dCTP (■), and dGTP (▽) pools versus time of exposure. No adenosine deaminase inhibitor was used for this experiment. Note that the scale of the ordinate changes after the breakpoint. D, trypanosome proliferation without drug (■), with 0.5 mM deoxyadenosine (●), with 5 μM adenosine deaminase inhibitor coformycin (△), and with 0.5 μM deoxyadenosine + 5 μM coformycin (▽). Each curve is an average of two experiments.

The expansion of the dATP level leads to a rapid decrease in the dGTP pool, compatible with our in vitro data that dATP directs CDP and UDP reduction. However, the levels of dCTP and dTTP were unchanged rather than increased. Possibly, surplus dCTP and dTTP are degraded by the trypanosomes and excreted.

Deoxyadenosine efficiently blocked trypanosome proliferation in the presence of an inhibitor of the serum adenosine deaminase such as 5 μM coformycin (Fig. 4C) or 5 μM EHNA (9-erythro-2-hydroxy-3-nonyladenine) (data not shown). A possible mechanism for the toxicity of deoxyadenosine could be through the depletion of the dGTP pool. Unexpectedly, 5 μM coformycin (Fig. 4C) or EHNA (data not shown) alone also inhibited trypanosome proliferation, although not at all as efficiently as in combination with deoxyadenosine. This inhibition was not accompanied by any change in the dNTP pools (data not shown).

**DISCUSSION**

We have previously shown (13) that the *T. brucei* ribonucleotide reductase has two types of allosteric effector binding sites, namely specificity sites binding dATP, dGTP, and dTTP, and in addition, ATP with low affinity and activity sites binding dATP and ATP. The low affinity of ATP to the specificity sites is further clarified in the present study where we show that the binding is 5,000 times weaker than that of dATP. We assume that this binding is insignificant under physiological conditions because the ATP concentration is only 150 times higher than that of dATP. Instead, dATP directs pyrimidine nucleotide reduction in the trypanosome ribonucleotide reductase. The low affinity of ATP to the specificity sites suggests that the observed effects of ATP on enzyme activity are directed solely through the activity sites. Another new finding from the binding of nucleotide effectors was that dCTP has the capacity to bind the specificity site. However, the affinity as well as the effect on enzyme activity is low and similar to ATP, we believe that it is not relevant for the *in vivo* allosteric regulation of ribonucleotide reductase.

Based upon similarity in amino acid sequence, all the eu- karyotic class I ribonucleotide reductases have been believed to be allosterically regulated in a similar manner as the mammalian and *E. coli* class Ia enzymes (2). Our results showing that the *T. brucei* enzyme uses dATP rather than ATP to activate pyrimidine nucleotide reduction do not necessarily contradict this theory because low concentrations of dATP have been shown to stimulate CDP reduction in the *E. coli* and mammalian ribonucleotide reductases as well (5, 6). A comparison between the relative affinities of ATP and dATP to the specificity sites in relation to their physiological levels has not been made for *E. coli* or mammalian cells.

The other dNTPs seem to direct the specificity of the recombinant *T. brucei* ribonucleotide reductase in a manner similar to what has been described for other class I enzymes with dTTP-activating GDP reduction and dGTP-activating ADP reduction (2). Although we can fit the *T. brucei* ribonucleotide reductase specificity sites into a general model, the activity sites are unique. ATP activated the enzyme except when combined with dGTP. The different effects exerted by ATP, depending on the specificity effector, suggest a cross-talk between the two effector binding sites. As already pointed out ATP alone seems to be able to activate CDP reduction from the activity sites. This effect could be indirect, with empty specificity sites being CDP-specific and ATP bound to the activity site only increasing subunit interaction (8). However, we believe that the situation with empty specificity sites is of low physiological value because our estimated intracellular dATP concentration of 10 μM is close to the level that had a saturating effect on enzyme activity in our previous studies (13). Like ATP, dATP also had different effects depending on the status of the specificity sites, not affecting the dGTP-bound enzyme and activating the dTTP-bound enzyme. This is the first time that dATP bound to the activity site has been shown to activate enzyme activity.

Our results on the recombinant ribonucleotide reductase were largely supported by the *in vivo* dNTP pool measurements in trypanosomes grown in the presence of thymidine and deoxyadenosine as well as trypanosomes released from hydroxyurea inhibition. An exception is that in the deoxyadenosine-treated trypanosomes, the dCTP and dTTP pools did not increase as would be expected from the allosteric regulation of ribonucleotide reductase. We think that the majority of the ribonucleotide reductase molecules already are in the dATP-bound form under physiological conditions because the affinity is higher for dATP than the other effectors (13). Increased dATP levels are then unlikely to give any dramatic effects on CDP and UDP reduction.

An unexpected and interesting result from our NTP pool studies is the extremely low CTP content in trypanosomes. It is unclear why the trypanosomes keep this low level but for mammalian cells, it has been observed that the CTP level is important in the control of phospholipid synthesis (24, 25). On the nucleoside diphosphate level, CDP in particular but also UDP were much smaller than the purine NDP pools. In contrast, the concentration of pyrimidine dNTPs is high, consistent with our theory that most of the ribonucleotide reductase molecules are in a dATP-bound form under physiological conditions. The enzyme is further adapted to the low CDP pool by...
having a higher $K_{cat}/K_m$ for this substrate compared with the other substrates at optimal effector conditions.

Why then is the trypanosome ribonucleotide reductase not inhibited by dATP like the other class Ia enzymes? The ability to bind dATP with strong affinity to the activity sites suggests that the enzyme originates from a dATP-inhibited ancestor. The trypanosomes, having a shortage of pyrimidine nucleotide substrates, would gain from not being dATP-inhibited but instead use dATP to stimulate pyrimidine nucleotide reduction. A mutated mouse T-lymphoblast cell line was selected containing a ribonucleotide reductase that is not inhibited by dATP (5, 26). This mutation leads to elevated dNTP pools and an increased mutation rate. In agreement, the total dNTP pool in $T. brucei$ is higher than in the mouse fibroblasts we used as a control. However, the difference is not as dramatic as in the cells containing the activity site mutant, suggesting that the lack of inhibition by dATP can be compensated for by other mechanisms. In mouse and hamster fibroblasts, it was shown that one important mechanism is that nucleotides in excess are degraded and excreted into the culture medium (27, 28). The excretion is decreased if the deoxyribonucleotide synthesis is blocked by hydroxyurea, consistent with our results from trypanosomes grown in the presence of this drug. As soon as the smallest pool, dGTP, is used up for DNA synthesis, the other dNTP pools stabilize.

Hydroxyurea, deoxyadenosine, and the adenosine deaminase inhibitors coformycin and EHNA all inhibited trypanosome proliferation, although the last two compounds only had a partial effect. Unfortunately, hydroxyurea inhibits trypanosome proliferation at a similar IC$_{50}$ as mammalian cells, although they may be more accessible to the drug because they live in the bloodstream. Deoxyadenosine seems to block trypanosome proliferation by inhibiting GDP reduction. How ever, we were not able to check if this is the only mechanism, because the trypanosomes do not salvage deoxyguanosine. The inhibition of trypanosome proliferation by EHNA or coformycin alone is at the moment unclear. Unlike what might be expected from their function, the drugs did not influence the trypano some dATP or ATP pools.

We have shown that $T. brucei$, in contrast to mammalian cells, is unable to phosphorylate deoxyctidine and deoxyguanosine. Thymidine and deoxyadenosine, on the other hand, are readily taken up and incorporated into the dNTP pools, the latter in a totally unregulated manner. Although a thymidine kinase is present in most organisms studied, a specific deoxyadenosine kinase is highly unusual. Preliminary results indicate that deoxyadenosine is not taken up by an adenosine kinase because adenine is a poor competitor. The finding of a new deoxyribonucleoside kinase activity is interesting from a chemotherapeutical view and should facilitate the search for trypanocidal deoxyribonucleoside analogues.

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