Biochemistry

 Volume 26, Number 12 June 16, 1987

Perspectives in Biochemistry

Regulation of Deoxyribotide Synthesis†

Peter Reichard

Department of Biochemistry, Medical Nobel Institute, Karolinska Institutet, S-104 01 Stockholm, Sweden

Received January 30, 1987; Revised Manuscript Received March 13, 1987

Intracellular pools of the four deoxyribonucleoside triphosphates (dNTPs)[1] are limited in size. In quiescent mammalian cells that require dNTPs only for DNA repair, pools are often too small to be measured with accuracy. In cells engaged in DNA synthesis (S-phase cells) the smallest pool (usually dGTP) sufficies only for a few minutes or less of DNA synthesis (Walters et al., 1973; Skoog et al., 1973). One often finds considerable differences between the size of the four pools, and in extreme cases the dCTP pool may be up to 100 times larger than the dGTP pool even though equal amounts of each dNTP are consumed during DNA replication. Size relations between the four pools are important. A considerable body of evidence suggests that disturbances in the normal (albeit poorly defined) balance is mutagenic and also may lead to other genetic abnormalities (de Serres, 1985).

Regulation of dNTP synthesis occurs at two levels: (1) during the life cycle of the cell, since a major requirement for dNTPs exists only during S-phase, and (2) during S-phase, to ascertain that equal amounts of each of the four dNTPs are available for DNA replication. I will discuss both kinds of regulation, mainly as they take place in mammalian cells, but also include one mechanism recently discovered in *Escherichia coli* whose relevance for mammalian cells remains to be shown.

First, I shall discuss various aspects of the regulation of ribonucleotide reductase, an enzyme that occupies a key position for the production of dNTPs. This includes (1) the allosteric regulation of the enzyme, (2) the regulation of its content of tyrosyl free radical, and (3) the regulation of its synthesis. Thereafter, I will describe (4) the contribution of substrate cycles between deoxyribosides and their 5′-phosphates to the regulation of dNTP pools and finally (5) compartmentation of dNTP pools and, connected with this, channeling of dNTPs by multienzyme complexes.

(1) Allosteric Control Mechanisms. A single enzyme catalyzes the synthesis of the four dNTPs by reduction of the corresponding ribonucleotide (Thelander & Reichard, 1979). Regulation is achieved by complex allosteric properties of ribonucleotide reductases, which were discussed in depth in several previous reviews (Thelander & Reichard, 1979; Lammers & Follman, 1983; Sjöberg & Gräslund, 1983). Briefly, reductases contain two separate classes of allosteric binding sites for nucleoside triphosphates, one of which (the activity site) regulates the overall activity of the enzyme while the other (the specificity site) regulates its substrate specificity. dNTPs compete for binding to specificity sites, and thus the relative amount of dNTPs present in the cell regulates the specificity of the reductase for the four ribonucleotides. As an example, an increase in the dTTP pool would shift the specificity of the enzyme away from pyrimidine ribonucleotides toward reduction of guanine nucleotides.

Ribonucleotide reductase is not subject to any known regulation distinguishing between the specificity for the two pyrimidine ribonucleotides. Instead, the proper intracellular balance between dCTP and dTTP is maintained by enzymes that deaminate deoxycytidine nucleotides (Maley & Maley, 1972; Beck et al., 1975). The deaminases are stimulated by dCTP and inhibited by dTTP. The ultimate product (dTTP) thus shuts off its own synthesis and leads to accumulation of dCTP, redressing the balance between the two pyrimidine dNTPs.

The allosteric regulation of dNTP synthesis, originally deduced from work with isolated enzymes, is supported by a considerable amount of evidence from work with intact cells. Such experiments involve artificial perturbations of dNTP pools by addition of nucleosides (Klenow, 1959; Bjursell & Reichard, 1973) or inhibitors (Skoog & Nordenskjöld, 1971) and the effects of regulatory mutations of the reductase (Eriksson et al., 1981; Wadell & Ullman, 1983; Ayusawa et al., 1983). In particular, the characterization of separate mutations for the activity and specificity sites provides impressive evidence (Eriksson et al., 1981).

There should by now be little doubt that allosteric controls of both ribonucleotide reductase and dCMP (dCTP) de-

† This work was supported by grants from the Swedish Medical Research Council.

[1] Abbreviation: dNTP, deoxyribonucleoside triphosphate.

0006-2960/87/0426-3245$01.50/0

aminase play a major role in the regulation of dNTP synthesis. It is also worth noting that the same intricate interplay between various dNTPs for the regulation of both substrate specificity and overall activity of the reductase is maintained from *E. coli* to mammalian cells. Only reductases coded by some bacteriophages (Berglund, 1975) and herpes viruses (Lankinen et al., 1982) have lost part of these controls.

(*2*) *Regulation of the Free Radical of Ribonucleotide Reductase.* Ribonucleotide reductase consists of two nonidentical subunits, named proteins B1 and B2 for the *E. coli* enzyme (Thelander & Reichard, 1979) and proteins M1 and M2 (Thelander et al., 1980) for the enzyme from mammalian sources.

B2 and M2 can be isolated in an active and an inactive form. Active B2 and M2 contain an organic free radical, localized to a specific tyrosyl residue of the polypeptide chain (Sjöberg et al., 1978; Gräslund et al., 1982; Larsson & Sjöberg, 1986). The radical lacks one ring electron and thus is an oxidized tyrosyl residue. Inactive forms of B2 and M2 contain a normal tyrosine at the position of the radical in the polypeptide chain.

In addition to the tyrosyl radical both the active and inactive forms of B2 and M2 contain an iron center, with two antiferromagnetically coupled ferric ions linked by a μ-oxo bridge (Atkin et al., 1973; Petersson et al., 1980; Sjöberg et al., 1982). This center stabilizes the radical and is also involved in its generation.

Treatment of B2 or M2 with certain radical scavengers, notably hydroxyurea, destroys the tyrosyl radical (Ehrenberg & Reichard, 1972; Atkin et al., 1973) and gives rise to the inactive form of the enzyme (=B2/HU). *Anaerobic* incubation of B2 with an extract of *E. coli* has a similar inactivating effect (Barlow et al., 1983). In contrast, *aerobic* incubation of B2/HU with an *E. coli* extract results in the formation of the tyrosyl radical and active B2 (Barlow et al., 1983). The bacterial extract apparently contains two opposing activities that modify the radical content of B2 and thereby its catalytic activity.

The enzyme system that activates B2/HU by radical introduction has been purified and characterized (Eliasson et al., 1986; Fontecave et al., unpublished results). It consists of at least three proteins and requires reduced pyridine nucleotides, flavin, dithiothreitol, and oxygen. Two of the participating proteins were recently obtained in pure form and characterized as superoxide dismutase and an NAD(P)H: flavin oxidoreductase. A possible mechanism of radical introduction is discussed elsewhere (Eliasson et al., 1986). Here, I wish to point to the possibility that tyrosine modification—introduction of a free radical by the system described here and its destruction by an as yet poorly defined activity—may represent a way to regulate reductase activity in vivo.

(*3*) *Regulation of Enzyme Synthesis.* Extracts from quiescent mammalian cells contain little or no ribonucleotide reductase activity. After stimulation of DNA synthesis a dramatic increase in enzyme activity occurs, with the highest values in S-phase cells (Nordenskjöld et al., 1970; Elford et al., 1970). This depends on synthesis of new enzyme, but surprisingly, the synthesis of the two subunits (M1 and M2) constituting the active enzyme is not coregulated (Eriksson & Martin, 1981; Engström et al., 1985). Quiescent cells, e.g., lymphocytes or liver parenchymal cells, contain very low amounts of either subunit. After stimulation to enter the cell cycle, M1 is synthesized but its level then remains constant in cycling cells. In contrast, the amount of M2 varies throughout the cell cycle and peaks during S-phase. The half-life of M1 is 15 h while that of M2 amounts to only 3 h. DNA clones are now available for both subunits (Thelander & Berg, 1986), which should make it possible to identify regulatory signals and proteins and to distinguish between transcriptional and translational regulation.

Relevant in this connection are also results concerning the regulation of the synthesis of ribonucleotide reductase during embryogenesis (Standart et al., 1985, 1986). Unfertilized clam oocytes contain stores of M1 protein and M2 messenger RNA. Since this RNA is not translated, unfertilized eggs do not contain active ribonucleotide reductase. Fertilization provides the signal for the translation of the stored M2 messenger, and within a few minutes active reductase provides dNTPs for DNA replication.

In *E. coli* the genes for both subunits of ribonucleotide reductase form a single operon and are regulated together (Tuggle & Fuchs, 1986).

(*4*) *Substrate Cycles Involving Deoxyribotides.* In contrast to the various regulatory events described in the three previous sections, which concern the de novo synthesis of dNTPs, the mechanism discussed in this section deals with the interplay between degradation and resynthesis of preformed deoxyribotides.

Two inborn errors of metabolism in man provide clear-cut examples for the importance of catabolic reactions for the regulation of dNTP pools (Martin & Gelfand, 1981). Genetic loss of adenosine deaminase or purine nucleoside phosphorylase, two enzymes catabolizing purine deoxyribosides, leads to the accumulation of huge pools of dATP or dGTP and results in severe immunodeficiency diseases. Both enzymes are clearly required to maintain the balance between catabolic and anabolic events that set the proper level of the two purine dNTP pools.

The balance between synthesis and degradation is also important for the maintenance of pyrimidine dNTP pools. Experiments with tissue-cultured cells, involving isotope flow kinetics under steady-state conditions, demonstrated that pyrimidine deoxyribotides synthesized de novo continuously are degraded and excreted into the medium as deoxyribosides (Nicander & Reichard, 1985a,b). In exponentially growing 3T6 mouse fibroblasts as much as 28% dCDP synthesized by reduction of CDP was excreted. In the other direction, deoxyribosides from the medium enter cells and are used for dNTP synthesis. The balance between excretion and influx is set by intracellular substrate cycles (Nicander & Reichard, 1985b) that involve a deoxyribonucleotidase (Fritzon, 1978) dephosphorylating deoxyriboside 5′-phosphates and kinases (Maley & Maley, 1962; Durham & Ives, 1970) catalyzing the phosphorylation of deoxyribosides. The ratio between these two activities is shifted toward anabolism (and import of deoxyribosides) when dNTPs are short in supply, e.g., when de novo synthesis is blocked (Bianchi et al., 1986a). Catabolism (and excretion of deoxyribosides) is favored when dNTPs accumulate, e.g., when DNA synthesis is inhibited.

Substrate cycles provide a mechanism for the fine tuning of dNTP pools and adapt cells to the availability of extracellular deoxyribosides.

(*5*) *Compartmentation of dNTP Pools.* Conclusions drawn from pool measurements may be complicated by the possibility that a given nucleotide forms more than one kinetic or physical compartment. Pool measurements represent averages from a large number of cells. The size of dNTP pools varies considerably throughout the cell cycle, and in nonsynchronized populations, cells at different stages of the cycle will contain separate pools. Exponentially growing cultures are usually dominated by S-phase cells. Such cells contain larger dNTP

pools than cells not engaged in DNA replication, and pool measurements and pool kinetics will in this situation largely reflect on S-phase cells. Cell synchronization minimizes problems of intercellular compartments but may introduce other artifacts.

Compartmentation also occurs within one and the same cell. The dNTP pools of mitochondria form a separate intracellular compartment (Bestwick et al., 1982). Their small size makes this a minor problem, as long as one is not specifically interested in mitochondrial pools. Maybe of more general importance is the distribution of dNTPs between the cytoplasm and cell nucleus. Attempts to measure separate cytoplasmic and nuclear pools have given contradictory results (Skoog & Bjursell, 1974; Leeds et al., 1985). dNTPs are synthesized in the cytoplasm (Engström et al., 1984; Leeds et al., 1985) but used for DNA replication in the nucleus with no apparent permeation barrier for nucleotides. I would expect a rapid, essentially unidirectional flow of dNTPs from the cytoplasm into the nucleus. Their small pool sizes and rapid turnover make an experimental exploration of this question difficult.

For the moment I leave the specific problem of pool distribution between the cytoplasm and cell nucleus and return to the general question of intracellular compartmentation of dNTPs. I will discuss each dNTP separately. Compartmentation of dTTP was suggested first. The evidence came from isotope experiments that under varying conditions measured the incorporation of thymidine into DNA of intact mammalian cells (Fridland, 1973; Kuebbing & Werner, 1975). Unfortunately, possible fluctuations of the specific activity of dTTP were not taken into account. When in later experiments measurements of specific activities were included, the data fitted a single kinetic dTTP pool whose turnover was completely accounted for by its incorporation into DNA (Nicander & Reichard, 1983). Presently, I am not aware of any convincing data indicating compartmentation of dTTP.

There is, however, strong evidence for compartmentation of dCTP as well as of dGTP. For dCTP, isotope flow kinetics in 3T6 and 3T3 cells suggested that the dCTP pool fed by the action of ribonucleotide reductase differed from the pool labeled from radioactive deoxycytidine (Nicander & Reichard, 1983). The former pool was used preferentially for DNA replication, while the second pool served preferentially for the synthesis of lipodeoxynucleotides (G. Spyrou and P. Reichard, unpublished results), a process that takes place in the membrane fraction of cells. Communication between the two pools does occur, but is slow enough to permit detection of their different kinetic behavior.

Also dGTP appears to be compartmentalized. In S-49 cells part of the nucleotide pool is sequestered and is not in rapid equilibrium with dGTP feeding DNA replication (Nguyen & Sadée, 1986). The nature of the sequestered pool is not known. Interestingly, some evidence for sequestration of the ribonucleotides GTP and GDP (but not GMP) was also found. One possibility that should be considered as an explanation is the high affinity of GTP, GDP, and dGTP for guanine nucleotide binding proteins (Bourne, 1986).

For dATP, possible sequestering of the deoxynucleotide was suggested to explain some results concerning inhibition of DNA replication by hydroxyurea (Bianchi et al., 1986b). Also in this case, adventitious binding of the deoxynucleotide to proteins that normally bind ATP appears possible.

Compartmentation has been interpreted in terms of "channeling" of all four dNTPs, from their site of synthesis to the replication fork. According to this concept dNTP synthesis and dNTP consumption are linked within one large multienzyme complex, starting with ribonucleotide reductase and ending with DNA replicase. Within the complex, dNTPs are assumed to form a protected compartment, specially designed for DNA synthesis. Such a model was originally supported from work on DNA replication in T4 phage infected *E. coli* (Chiu et al., 1982; Allen et al., 1983). In its subsequent adaptation to mammalian cells (Reddy & Pardee, 1980, 1982) it was suggested that the multienzyme complex (the replitase) only exists in S-phase cells during DNA replication. Experiments cited in favor of this model were (1) a migration of the appropriate enzymes from the cytoplasm into the nucleus at the G1/S boundary, (2) the preferential use of ribonucleotides over deoxyribonucleotides for DNA replication in permeabilized cells, and (3) a complete shutoff of dNTP synthesis after inhibition of DNA replication, explainable by protein–protein interaction (Reddy & Pardee, 1983). The replitase model has become quite popular since it makes compartmentation easily understandable. Unfortunately, much later work contradicts the model: (1) during the entire cell cycle ribonucleotide reductase remains in the cytoplasm (Engström et al., 1984; Leeds et al., 1985) while DNA polymerase resides in the cell nucleus (Bensch et al., 1982); (2) channeling of ribonucleotides into DNA is doubtful (Spyrou & Reichard, 1983; Pawlak et al., 1986); and (3) inhibition of DNA replication does only partially turn off dNTP synthesis and is explainable by allosteric effects (Nicander & Reichard, 1985a).

In view of these negative findings, the replitase model can hardly be accepted in its original form. This does of course not negate the existence of multiprotein complexes in connection with DNA replication, for which there is much evidence (Kornberg, 1980). Some form of channeling of small molecules during the synthesis of dNTPs in mammalian cells remains an attractive possibility, albeit difficult to prove. One recent speculation (Mathews & Slabaugh, 1986) suggests that the rough endoplasmic reticulum associated with the nuclear membrane might serve as a vehicle for transport of newly synthesized cytoplasmic dNTPs into the nucleus. Be that as it may, we should be careful not to invoke channeling just as an easy way to explain compartmentation of dNTPs and forget to look for other experimentally verifiable explanations.

References

Allen, J. R., Lasser, G. W., Goldman, D. A., Booth, J. W., & Mathews, C. K. (1983) *J. Biol. Chem. 258*, 5746–5753.

Atkin, C. L., Thelander, L., Reichard, P., & Lang, G. (1973) *J. Biol. Chem. 248*, 7464–7472.

Ayusawa, D., Iwata, K., & Seno, T. (1983) *Cancer Res. 43*, 814–818.

Barlow, T., Eliasson, R., Platz, A., Reichard, P., & Sjöberg, B.-M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1492–1495.

Beck, C. F., Eisenhardt, A. R., & Neuhard, J. (1975) *J. Biol. Chem. 250*, 609–611.

Bensch, K. G., Tanaka, S., Hu, S.-Z., Wang, T. S.-F., & Korn, D. (1982) *J. Biol. Chem. 257*, 8391–8396.

Berglund, O. (1975) *J. Biol. Chem. 250*, 7450–7455.

Bestwick, R. K., Moffett, G. L., & Mathews, C. K. (1982) *J. Biol. Chem. 257*, 9300–9304.

Bianchi, V., Pontis, E., & Reichard, P. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 986–990.

Bianchi, V., Pontis, E., & Reichard, P. (1986b) *J. Biol. Chem., 261*, 16037–16042.

Bjursell, G., & Reichard, P. (1973) *J. Biol. Chem. 248*, 3904–3909.

Bourne, H. R. (1986) *Nature (London) 321*, 814–816.

Chiu, C. S., Cook, K. S., & Greenberg, G. R. (1982) *J. Biol. Chem. 257*, 15087–15097.

de Serres, F. J., Ed. (1985) in *Genetic Consequences of Nucleotide Pool Imbalance*, Plenum, New York.
Durham, J. P., & Ives, D. H. (1970) *J. Biol. Chem. 245*, 15021–15027.
Ehrenberg, A., & Reichard, P. (1972) *J. Biol. Chem. 247*, 3485–3488.
Elford, H. L., Freese, M., Passami, E., & Morris, H. P. (1970) *J. Biol. Chem. 245*, 5228–5233.
Eliasson, R., Jörnvall, H., & Reichard, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2373–2377.
Engström, Y., Rozell, B., Hansson, H.-A., Stemme, S., & Thelander, L. (1984) *EMBO J. 3*, 863–867.
Engström, Y., Eriksson, S., Jildevik, I., Skog, S., Thelander, L., & Tribukait, B. (1985) *J. Biol. Chem. 260*, 9114–9116.
Eriksson, S., & Martin, D. W., Jr. (1981) *J. Biol. Chem. 256*, 9436–9440.
Eriksson, S., Gudas, L. J., Clift, S. M., Caras, J. W., Ullman, B., & Martin, D. W., Jr. (1981) *J. Biol. Chem. 256*, 10193–10197.
Fridland, A. (1973) *Nature (London), New Biol. 243*, 105–107.
Fritzon, P. (1978) *Adv. Enzyme Regul. 16*, 43–61.
Gräslund, A., Ehrenberg, A., & Thelander, L. (1982) *J. Biol. Chem. 257*, 5711–5715.
Klenow, H. (1959) *Biochim. Biophys. Acta 35*, 412–421.
Kornberg, A. (1980) *DNA Replication*, Freeman, San Francisco.
Kuebbing, D., & Werner, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3333–3336.
Lammers, M., & Follman, H. (1983) *Struct. Bonding (Berlin) 54*, 27–91.
Lankinen, H., Gräslund, A., & Thelander, L. (1982) *J. Virol. 41*, 893–900.
Larsson, Å., & Sjöberg, B.-M. (1986) *EMBO J. 5*, 2037–2040.
Leeds, J. M., Slabaugh, M. B., & Mathews, C. K. (1985) *Mol. Cell Biol. 5*, 3443–3450.
Maley, F., & Maley, G. F. (1962) *Biochemistry 1*, 847–851.
Maley, F., & Maley, G. F. (1972) *Curr. Top. Cell Regul. 5*, 177–228.
Martin, D. W., Jr., & Gelfand, E. W. (1981) *Annu. Rev. Biochem. 50*, 845–877.
Mathews, C. K., & Slabaugh, M. B. (1986) *Exp. Cell Res. 162*, 285–295.
Nguyen, B. T., & Sadée, W. (1986) *Biochem. J. 234*, 263–269.
Nicander, B., & Reichard, P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1347–1351.
Nicander, B., & Reichard, P. (1985a) *J. Biol. Chem. 260*, 5376–5381.
Nicander, B., & Reichard, P. (1985b) *J. Biol. Chem. 260*, 9216–9222.
Nordenskjöld, B. A., Skoog, L., Brown, N. C., & Reichard, P. (1970) *J. Biol. Chem. 245*, 5360–5368.
Pawlak, K., Lawi-Berger, C., & Sadée, W. (1986) *Biochem. J. 238*, 13–21.
Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M., & Reichard, P. (1980) *J. Biol. Chem. 255*, 6706–6712.
Reddy, G. P. V., & Pardee, A. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3312–3316.
Reddy, G. P. V., & Pardee, A. B. (1982) *J. Biol. Chem. 257*, 5711–5715.
Reddy, G. P. V., & Pardee, A. B. (1983) *Nature (London) 304*, 86–88.
Sjöberg, B.-M., & Gräslund, A. (1983) *Adv. Inorg. Biochem. 5*, 87–110.
Sjöberg, B.-M., Reichard, P., Gräslund, A., & Ehrenberg, A. (1978) *J. Biol. Chem. 253*, 6863–6865.
Sjöberg, B.-M., Loeher, T. M., & Sanders-Loehr, J. (1982) *Biochemistry 21*, 96–102.
Skoog, L., & Nordenskjöld, B. (1971) *Eur. J. Biochem. 19*, 81–89.
Skoog, L., & Bjursell, G. (1974) *J. Biol. Chem. 249*, 6434–6438.
Skoog, K. L., Nordenskjöld, B. A., & Bjursell, K. G. (1973) *Eur. J. Biochem. 33*, 428–432.
Spyrou, G., & Reichard, P. (1983) *Biochem. Biophys. Res. Commun. 115*, 1022–1026.
Standart, N. M., Bray, S. J., George, E. L., Hunt, T., & Ruderman, J. V. (1985) *J. Cell Biol. 100*, 1968–1976.
Standart, N., Hunt, T., & Ruderman, J. V. (1986) *J. Cell Biol. 103*, 2129–2136.
Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133–158.
Thelander, L., & Berg, P. (1986) *Mol. Cell. Biol. 6*, 3433–3442.
Thelander, L., Eriksson, S., & Åkerman, M. (1980) *J. Biol. Chem. 255*, 7426–7432.
Tuggle, C. K., & Fuchs, J. A. (1986) *EMBO J. 5*, 1077–1085.
Wadell, D., & Ullman, B. (1983) *J. Biol. Chem. 258*, 4226–4231.
Walters, R. A., Tobey, R. A., & Ratliff, R. L. (1973) *Biochim. Biophys. Acta 319*, 336–347.