THE COBAMIDE-DEPENDENT RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE OF LACTOBACILLI

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Ribonucleotide reduction in Escherichia coli has been the subject of extensive investigations by Reichard and his collaborators who have shown that in this organism an enzyme is present which reduces ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates (Reichard, 1962; Larsson, 1963). The enzyme requires ATP and Mg^{2+} for activity and uses reduced lipoic acid as a reductant, although evidence has been presented that the physiological reductant for this reaction is the reduced form of thioredoxin, a protein of molecular weight around 12,000 which, in the oxidized form, contains one disulfide bond per molecule (Laurent, Moore and Reichard, 1964). Thioredoxin is reduced by NADPH in the presence of reductase which has been extensively purified from E. coli (Moore, Reichard and Thelander, 1964). A ribonucleoside diphosphate reductase system is reported to be present also in Novikoff hepatoma cells which is capable of utilizing reduced lipoate, chemically reduced

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bacterial thioredoxin or a combination of NADPH, bacterial thioredoxin and bacterial thioredoxin reductase (Moore and Reichard, 1964). In these reductase systems no evidence has been found for the participation of cobamides.

In contrast to the system in E. coli, the reductase present in Lactobacillus leichmannii is active only in the presence of 5'-deoxyadenosylcobalamin (Blakley and Barker, 1964), which acts as a freely dissociable coenzyme with an apparent K_m of 9×10^{-7}M (Blakley, 1965). In unfractionated bacterial extracts, the reductase was found to catalyse the reduction of several ribonucleoside mono-, di- and triphosphates in the presence of reduced lipoic acid, ATP and Mg^{2+}, but di- and triphosphates were better substrates than the corresponding monophosphates. The ability of reduced lipoic acid to act as a reductant has also been reported by Abrams and Duraiswami (1965) who have also shown that the bond between the base and pentose is not broken during the enzymic reduction.

The reductase has now been partially purified by chromatography on hydroxyapatite, on DEAE-Sephadex and on Sephadex. In the initial stages of purification the reductase lost most of its ability to reduce CDP in the reaction mixture previously described (Blakley, 1965), although it was still fully active with CTP. At the same time it was noticed that the ATP present in the reaction mixture was being reduced to an adenine deoxyribonucleotide. These results suggested that the substrate for the reductase is in fact CTP rather than CDP and that in the presence of crude extracts CDP had been converted to CTP by an ATP:CDP phosphotransferase, which had been removed by the purification procedure. It also seemed likely that ATP and possibly

other ribonucleoside triphosphates are substrates for the enzyme.

To obtain further evidence regarding the substrate specificity of the reductase, the enzyme has been further purified and its activity investigated by the use of a colorimetric assay procedure which employs the diphenylamine reagent (to be published). Table I shows the results on substrate specificity obtained with the purified enzyme. It may be seen that the reductase shows much greater activity with GTP than with any other substrate tested, but has significant activity with all the other triphosphates. However, UTP was much more slowly reduced than the other triphosphates tested. Diphosphates also appear to be able to act as substrates but reduction of the diphosphate proceeds five to ten times more slowly than the reduction of the corresponding triphosphate. It is possible that some

TABLE I

Substrate specificity of ribonucleotide reductase from *L. leichmannii*

Substrate	Deoxyribonucleotide synthesized, mμmoles	Substrate	Deoxyribonucleotide synthesized, mμmoles
AMP	0	ITP	162
ADP	26	CMP	8
ATP	167	CDP	24
GMP	3	CTP	121
GDP	116	UMP	0
GTP	1,200	UDP	7
IMP	10	UTP	35

Reaction mixtures containing 10 mM ribonucleotide, 30 mM reduced lipoic acid, 0.1 M potassium phosphate buffer, pH 7.3, 8 μM 5'-deoxyadenosylcobalamin and 0.096 mg of enzyme were incubated under nitrogen for 30 minutes before colorimetric assay of deoxyribonucleotide.

of the commercial samples of ribonucleoside diphosphates were contaminated with the corresponding triphosphates and that these contaminating triphosphates were being reduced when the diphosphates appeared to be acting as substrates. However, the amount of deoxyribonucleotide formation from diphosphates seems too large for this to be likely.

The L. leichmannii enzyme is thus more properly termed a ribonucleoside triphosphate reductase. Although it has not been demonstrated that the products are deoxyribonucleoside triphosphates, the absence of any requirement for coenzymes other than the cobamide suggests that this is the case. If this is so, this enzyme functions in a more efficient manner than the E. coli enzyme since it forms deoxyribonucleoside triphosphates required for DNA synthesis as its immediate products. The low activity with UDP and UTP is also useful to the cell since it obviates the production of unwanted uracil deoxyribonucleotides in any significant amount.

The results reported in Table I were obtained with reaction mixtures from which Mg^{2+} was omitted and in which ATP was present only when it was itself the substrate to be reduced. Addition of ATP and Mg^{2+} did not accelerate reduction of other ribonucleotides by the purified enzyme and it is therefore concluded that the requirement for ATP and Mg^{2+} previously reported must be entirely connected with the generation (or regeneration) in unfractionated extracts of ribonucleoside triphosphates from the corresponding di- and monophosphates. The L. leichmannii reductase reported here thus differs sharply from the E. coli enzyme which requires ATP and Mg^{2+} even

after considerable purification (Reichard, 1962; Moore and Reichard, 1963).

While the requirement for ATP and Mg^{2+} is lost during purification of the L. leichmannii enzyme, the requirement for 5'-deoxyadenosylcobalamin is retained and reduced lipoic acid continues to function as reductant (Table II).

TABLE II

Requirements for ribonucleoside triphosphate reductase

Reaction mixture	Deoxyribonucleotide synthesized, mμmoles
Complete	1,200
Deoxyadenosylcobalamin omitted	9
Reduced lipoate omitted	13
GTP omitted	9
Boiled enzyme	13

Reaction mixtures contained the same components as in Table I with GTP as the ribonucleotide substrate, except as indicated. The procedure was the same as in Table I.

The L. leichmannii enzyme also appears to differ from the E. coli reductase in that NADPH does not act as the ultimate reductant for the former even in unfractionated bacterial extracts (Blakley, 1965). This suggests that the lactobacilli do not contain a thioredoxin-thioredoxin reductase system. Thioredoxin is unlikely to be present in the purified L. leichmannii reductase preparation since the latter has been subjected to gel filtration on Sephadex G200 which separated the reductase from proteins of molecular weight 12,000 (the value reported for E. coli thioredoxin) by a wide margin. A procedure similar to that of Leach and O'Shea (1965) gave results indicating a molecular weight for the reductase of about 25,000.

Investigations of the distribution of the cobamide-dependent ribonucleoside triphosphate reductase have indicated that it is present in a number of lactobacilli. In addition to L. leichmannii ATCC 4797 and 7830, extracts of the following strains of L. acidophilus contain the reductase: ATCC 314, 832, 4356, 4357, 4796 and 4962. In extracts from a number of other lactobacilli and in Aerobacter aerogenes and Clostridium tetanomorphorum, however, the enzyme could not be detected. It has also been found that in the bone marrow of rabbits rendered anemic by phenylhydrazine administration there was no detectable activation of the reduction of ribonucleotides by 5'-deoxyadenosylcobalamin. Nevertheless, it should be noted that in L. leichmannii the reductase appears to be present in an amount considerably in excess of that required to synthesize deoxyribonucleotides at a rate necessary to keep pace with DNA synthesis (Blakley, 1965). In cells which reproduce at a much slower rate than L. leichmannii an amount of the reductase several orders of magnitude less than that present in L. leichmannii would therefore be sufficient for the needs of the cell. Such small amounts of a cobamide-dependent reductase might not have been detected by the methods we have employed so that in some of the organisms where the reductase could not be detected it may be present in small but adequate amounts. In this regard it is relevant that in most of these organisms, and particularly in bone marrow, the rate of ribonucleotide reduction (under all the experimental conditions which have been used) was very low compared with that obtained with unfractionated L. leichmannii extracts.

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REFERENCES

Abrams, R. and Duraiswami, S., Biochem. Biophys. Res. Communs., 18, 409 (1965).
Blakley, R.L., J. Biol. Chem., 240, 2173 (1965).
Blakley, R.L. and Barker, H.A., Biochem. Biophys. Res. Communs., 16, 391 (1964).
Larsson, A., J. Biol. Chem., 238, 3414 (1963).
Leach, A.A. and O'Shea, P.C., J. Chromat., 17, 245 (1965).
Moore, E.C. and Reichard, P., J. Biol. Chem., 238, PC2244 (1963).
Moore, E.C. and Reichard, P., J. Biol. Chem., 239, 3453 (1964).
Moore, E.C., Reichard, P. and Thelander, L., J. Biol. Chem., 239, 3445 (1964).
Reichard, P., J. Biol. Chem., 237, 3513 (1962).