HYDROGEN-DONOR SPECIFICITY OF RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE FROM LACTOBACILLUS LEICHMANNII

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Received November 2, 1965

A cobamide coenzyme-dependent ribonucleotide reductase was first reported in cell-free extracts of Lactobacillus leichmannii by Blakley and Barker (1). In these extracts, reduction of ribonucleotides in the presence of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and 2-mercaptoethanol was detected by a radioisotopic assay. As neither mercaptoethanol nor reduced pyridine nucleotides alone would stimulate ribonucleotide reduction, the ultimate hydrogen donor in the system appeared to be glucose-6-phosphate, although the detailed reaction mechanism remains obscure. Subsequently, it was found (2) that reduced lipoic acid could completely replace the requirement for both the glucose-6-phosphate system and mercaptoethanol, stimulating the rate of ribonucleotide reduction by crude extracts several hundred-fold. Reduced pyridine nucleotides, as well as a number of mono- and dithiols could not replace dihydrolipoate (2).

With dihydrolipoate as hydrogen donor, the reaction rates have been sufficiently high to permit the use of the diphenylamine colorimetric method (3) for the assay of deoxyribonucleotide production. Using this more rapid and convenient assay method, the substrate specificities of the enzyme have been investigated with purified enzyme preparations. It has been concluded (4) that the

L. leichmannii enzyme is a ribonucleoside triphosphate reductase. This communication presents further evidence on the hydrogen donor specificity of the enzyme.

Cells of L. leichmannii were grown, harvested, and lysed as described previously (2). The ribonucleoside triphosphate reductase was purified from the crude cell-free extracts by treatment with protamine sulfate, fractionation with ammonium sulfate, and column chromatography on hydroxylapatite and C_{γ}-alumina (mixed with cellulose to increase the flow rate). The combination of these steps achieved a 70 to 100-fold purification of the enzyme as compared with the crude cell-free extracts.

Table I shows the rate of ATP reduction by the purified enzyme in the presence of various thiols as hydrogen donors. The monothiol, 2-mercaptoethanol, was completely inactive. Other monothiols, such as cysteine and reduced glutathione, were also

TABLE I

Thiol Specificity for ATP Reduction

Thiol	Thiol Concentration (mM)	ATP reduced (mμmoles)
Dihydrolipoic acid	20	132
Dihydrolipoyl-glycine	20	59
Dihydrolipoyl-glycylglycine	20	73
Dihydrolipoyl-L-lysine	4*	16
1,3-Dithiopropan-2-ol	20	72
1,3-Dithiopropane	5*	21
2,3-Dithiopropanol	20	0
Dithioerythrol	20	72
Dithiothreitol	20	71
2-Mercaptoethanol	20	0

*Concentration limited by low solubility

Reaction mixtures contained 100 mM potassium phosphate, pH 7.3, 10 mM ATP, 2 mM EDTA, 8 μM 5'-deoxyadenosylcobalamin, 25 μg of purified L. leichmannii enzyme, and the indicated amount of thiol in a total volume of 0.5 ml. Incubations were performed under nitrogen at 37°C for 20 minutes, and the deoxyribonucleotide formed was determined by the colorimetric assay using deoxyadenosine monophosphate as a standard.

inactive, as has been reported previously (2). Of the dithiols tested, only 2,3-dithiopropanol was inactive. The remainder of the dithiols in Table I, all of which were active, contain either 6,8-, 1,3-, or 1,4-dithiol groups and differ from the inactive 2,3-dithiopropanol in that they can undergo an intramolecular cyclization upon oxidation. The rate of ATP reduction varies substantially, depending upon the dithiol present. Under the experimental conditions described in Table I, dihydrolipoate is considerably more active than the dihydrolipoyl-amino acids, suggesting that a protein-bound lipoic acid may not be the physiological hydrogen donor in the reductase reaction. The propane and butane dithiol derivatives are also less active than dihydrolipoate under the experimental conditions employed. It should be emphasized, however, that the maximal velocity for each dithiol compound shows a different dependence upon pH and concentration. This may in part be due to the variation in pK values of the sulfhydryl groups of the several compounds. In general, the dithiol concentrations required for maximal velocity are rather high (approximately 50 mM at pH 7.5 for dihydrolipoate), and preliminary results from plots of reaction velocity vs. dithiol concentration indicate that the reaction does not appear to follow simple kinetics.

In experiments using the purified enzyme, it has also been possible to follow the reaction continuously by direct spectrophotometric measurement of dihydrolipoic acid oxidation. In order to obtain measurable changes in absorbancy, larger amounts of enzyme were used in these experiments, and ATP was replaced by GTP as substrate, since the latter is reduced by the enzyme at a considerably faster rate (4). Under the experimental conditions described in Table II, the oxidation of dihydrolipoate was entire-

TABLE II

Dihydrolipoic Acid Oxidation and GTP Reduction by L. leichmannii Ribonucleotide Reductase

Expt.	Enzyme (μg)	Incubation time (min)	Dihydrolipoate oxidized (mμmoles)	GTP reduced (mμmoles)
1	392	5	554	543
2	224	10	547	612
3	94	15	332	381

Cuvettes containing 100 mM potassium phosphate, pH 7.5, 2 mM EDTA, 10 mM GTP, 20 mM dihydrolipoate, 8 μM 5'-deoxyadenosylcobalamin, and enzyme as indicated in a total volume of 0.5 ml, were briefly flushed with nitrogen, covered, and the change in absorbancy recorded at 333 mμ for the periods indicated on a Cary Model 14 spectrophotometer. The reference cuvette was identical, except for the omission of GTP. After incubation, aliquots were taken from the cuvettes for colorimetric determination of deoxyribonucleotide formed.

ly dependent upon the presence of enzyme, cobamide coenzyme, and GTP. The oxidation rate was linear for at least 15 minutes, and the amount of dihydrolipoate oxidized corresponded closely to the amount of deoxyribotide produced (Table II). These results demonstrate the direct relationship between dihydrolipoic acid oxidation and deoxyribonucleotide synthesis.

The ribonucleotide reductase of L. leichmannii differs markedly from the CDP reductase system found in E. coli (5,6), in that the former enzyme is strictly cobamide coenzyme-dependent; it preferentially reduces ribonucleoside triphosphates, rather than diphosphates; and it does not require the presence of magnesium ions for activity. Dihydrolipoic acid can serve as the hydrogen donor to both enzyme systems, although reduced thioredoxin has been identified as the actual hydrogen donor for the E. coli system under physiological conditions. Thioredoxin is a protein with a molecular weight of 12,000 and contains one molecule of cystine, the S-S bond of which can readily undergo oxidation-

reduction (7). In E. coli, it is reduced by NADPH in the presence of thioredoxin reductase. Although it has not been possible to demonstrate NADPH-dependent or NADH-dependent ribonucleotide reduction with the L. leichmannii enzyme, it was of considerable interest to determine whether reduced thioredoxin of E. coli could act as a hydrogen donor to the ribonucleoside triphosphate reductase of L. leichmannii.

Thioredoxin and thioredoxin reductase were prepared from E. coli B as described by Reichard and his colleagues (7,8). The thioredoxin was enzymatically reduced with NADPH and E. coli thioredoxin reductase in the presence of the purified ribonucleotide reductase of L. leichmannii. Table III summarizes the results of

TABLE III

Reduced Thioredoxin as a Hydrogen Donor for ATP Reduction by L. leichmannii Ribonucleotide Reductase

Omissions	ATP reduced (mμmoles)
None	71.5
NADPH	1.0
Thioredoxin	1.2
Thioredoxin + thioredoxin reductase	0.3
Cobamide coenzyme	0.0
Ribonucleotide reductase	0.0

The complete assay system contained, in a total volume of 0.5 ml, 100 mM potassium phosphate, pH 7.3, 10 mM ATP, 2 mM EDTA, 8 μM 5'-deoxyadenosylcobalamin, 25 μg of ribonucleotide reductase, 1 mM NADPH, 0.06 mM thioredoxin, and 25 μg of thioredoxin reductase. The conditions of incubation and the method of deoxyribonucleotide estimation were the same as described in Table I.

these experiments. It is clear that reduced thioredoxin can indeed act as a hydrogen donor to the L. leichmannii enzyme. As shown in the Table, the reaction remains strictly dependent upon the presence of cobamide coenzyme. It should be noted that the concentration of thioredoxin used in these experiments (Table

III) is a small fraction (3×10^{-3}) of the dihydrolipoate concentration normally used in standard assays of the enzyme (Table I). Thus reduced thioredoxin is a far more efficient hydrogen donor in the ribonucleoside triphosphate reductase reaction than dihydrolipoate.

Because the enzyme requires relatively high concentrations of dihydrolipoate and generally shows rather low specificity toward dithiols, facts which appear to argue against dihydrolipoic acid as the physiological hydrogen donor in L. leichmannii, we have considered the possibility that there also may be a thioredoxin-like protein present in this organism. Preliminary investigations have been restricted to efforts to detect thioredoxin-like components in the crude cell-free extracts. Catalytic amounts of thioredoxin can easily be detected by measuring NADPH oxidation spectrophotometrically in the presence of thioredoxin reductase and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTN) or insulin, as reduced thioredoxin readily and non-specifically reduces the disulfide bond of DTN or those of insulin (7). Experiments using extracts of L. leichmannii and the purified thioredoxin reductase of E. coli have failed to detect any DTN or insulin-dependent NADPH (or NADH) oxidation. Similarly, no deoxyribonucleotide synthesis has been detected by supplementing the purified ribonucleoside triphosphate reductase with crude extracts of L. leichmannii, NADPH, and thioredoxin reductase from E. coli. These experiments do not, of course, conclusively exclude the possibility of a thioredoxin-like protein in L. leichmannii, as they would not have detected such material present in very small amounts or a protein more labile than the E. coli thioredoxin. Moreover, a thioredoxin-like compound, if present in L. leichmannii, may not necessarily be reduced by the thioredoxin reductase from E. coli and may have

thus escaped detection by the experiments described above.

Positive identification of the physiological hydrogen donor for the ribonucleoside triphosphate reductase system in L. leichmannii will necessarily have to await isolation of such a donor from the organism itself and perhaps elucidation of the detailed reaction mechanism for the enzyme system. However, the experiments presented here indicate that the most effective hydrogen donor yet investigated is E. coli thioredoxin, a molecule of considerable size, which nevertheless reacts rapidly at very low concentrations.

ACKNOWLEDGEMENTS

We are indebted to Mr. P. Allen and Mr. M.A. de Smet for technical assistance; to Dr. Lester J. Reed for gifts of D,L-lipoyl-glycylglycine and N^{ε}-D,L-lipoyl-L-lysine, and to Dr. E. Lester Smith for a gift of 5'-deoxyadenosylcobalamin.

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