Purification and Properties of Ribonucleotide Reductase from Leukemic Mouse Spleen*

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SUMMARY

A 13-fold increase was observed in the specific activity of ribonucleotide reductase activity in the spleen of mice infected with a murine leukemia virus. After partial purification, the properties of the enzyme were investigated. The pH optimum was between 6.8 and 7.4. There was an absolute requirement for a hydrogen donor, dithiothreitol. Magnesium ions stimulated the reaction. The ribonucleotide diphosphates were the preferred substrates. The reduction rate was affected by the presence of nucleoside triphosphates in concentrations ranging from $5 \times 10^{-5}$ to $10^{-3}$ M. The reduction of CDP was stimulated by dCTP and ATP. UDP reduction was stimulated by ATP. The reduction of ADP was stimulated by GTP and dGTP. The GDP reduction was stimulated by dTTP. In the presence of ATP, the weak activators, dTTP and dATP, inhibit the CDP reduction. With minor exceptions, the ribonucleotide reductase from murine leukemic spleen closely resembles that of Novikoff hepatoma.

Regulation of the enzymatic reduction of ribonucleotides to deoxyribonucleotides has been investigated in several microbial and animal systems. These include Escherichia coli (1), Lactobacillus leichmanii (2, 3), the Novikoff rat hepatoma (4), and embryonic tissues from the chick and rat (5, 6). It has been established that this reduction, catalyzed by ribonucleotide reductase, is generally regulated through the concentration of nucleotides, which act as allosteric effectors (7-10). The fact that deoxyribonucleotides are present only in very small amounts in cells (11) suggests that the reduction of ribonucleotides to deoxyribonucleotides would be a suitable locus for the regulation of deoxyribonucleic acid formation (5, 12). In view of the probable importance of this reaction in cell proliferation, and the potential value of this reaction as a target site for antimetabolites, it seemed pertinent to determine the levels of the enzyme and regulation of this system in the course of viral leukaemogenesis. Changes in ribonucleotide reductase have been observed following the infection of bacteria (13, 14) and animal cells with viruses. A 3- to 5-fold increase in activity occurs upon polyoma virus infection of contact-inhibited mouse kidney cells (15). The ribonucleotide reductase level in cell-free extracts of Yaba Pox virus tumor is from 2- to 10-fold higher than the levels found in normal monkey tissues (16). The present paper reports a striking increase in ribonucleotide reductase activity in the spleen of mice following infection with a murine leukemia virus and describes some properties of the partially purified enzyme. The results show that the reduction occurs mainly at the diphosphate level and requires a sulfhydryl compound as a hydrogen donor. Although the enzyme resembles the E. coli (17) and the Novikoff hepatoma (18) ribonucleotide reductases in many respects, several differences were found in the allosteric effectors regulating this system in the leukemic mouse spleen.

MATERIALS AND METHODS

Preparation of Enzyme—Female Swiss mice, weighing approximately 25 g, were inoculated intraperitoneally with 0.2 ml of a cell-free extract prepared from spleens of DBA/2 mice, which had been inoculated with Friend murine leukemia virus (19). Control animals were injected with an equal volume of 0.15 M NaCl or with 1 mg of phenylhydrazine in 0.2 ml of 0.15 M NaCl. The spleens from the virus-infected animals weighed up to 20 times more than spleens of normal controls. The administration of phenylhydrazine, which causes a hemolytic anemia in the mouse, results in a 2- to 3-fold increase in splenic weight. Groups of six to 10 animals were killed at various time intervals. The enzyme was purified from the spleen of animals killed 5 days postinoculation. Ten spleens, weighing a total of 14 g were pooled and homogenized in an equal volume of 0.02 M Tris-HCl buffer, pH 7.0, in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 20,000 \times g for 45 min. The supernatant fluid was passed through a column (2.4 \times 40 cm) of Sephadex G-25 to eliminate endogenous nucleotides and eluted with the same Tris HCl buffer. Streptomycin sulfate was added to the eluate to a final concentration of 1% (w/v) and the precipitate was removed by centrifugation. An ammonium sulfate precipitation step followed. The precipitate formed after the addition of ammonium sulfate (1.95 g to 10 ml) was removed by centrifugation. An additional 0.92 g of ammonium sulfate was added to the supernatant fluid. The precipitate, which represents a 25 to 40%
FIG. 1. Specific activity of ribonucleotide reductase in mouse spleen after infection with murine leukemia (Friend). •—•, specific activity. - - - - X, spleen weight.

Table I
Purification of ribonucleotide reductase from murine leukemic spleen

| Step                                           | Volume | Protein | Total activity | Specific activity |
|-----------------------------------------------|--------|---------|----------------|------------------|
| Crude extract                                 | 12     | 840     | 11.8           | 0.014            |
| Sephadex G-25 chromatography                   | 25     | 675     | 44.6           | 0.066            |
| 1% streptomycin sulfate supernatant            | 24     | 504     | 56.5           | 0.112            |
| 25 to 40% ammonium sulfate precipitate         | 2.5    | 29      | 29.3           | 1.00             |

saturated ammonium sulfate fraction, was dissolved in 1 ml of 0.02 M Tris-Cl buffer, pH 7.0. The solution was passed through a column, 1 x 30 cm, of Sephadex G-25 which was eluted with the same Tris buffer. All procedures were performed at 4°C. The enzyme solution was used immediately or kept at -60°C for up to 1 week.

Enzyme Assay Method—The assay method for ribonucleotide reductase in our laboratory was described previously (20). The assay mixture, in a final volume of 120 μl, contained 1 μmole of potassium phosphate buffer (pH 7.0), 250 μmole of ATP, 50 μmole of magnesium acetate, 5 μmole of ferric chloride, 750 μmole of dithiothreitol, 20 μmole of cytidine 5'-diphosphate-2H, and approximately 0.7 mg of protein. In some experiments, 20 μmole of UDP-2H, ADP-2H, GDP-2H, CTP-2H, or CMP-2H were substituted for CDP-2H. This mixture was incubated at 37°C for 15 min. After centrifugation, the supernatant solution was treated with 10 μl of bacterial alkaline phosphatase (34 units per ml) to degrade nucleotides to nucleosides. Deoxycytidine and deoxyuridine were separated from cytidine, uridine, cytosine, and uracil by two-dimensional descending paper chromatography on Whatman No. 1 paper. The first solvent system used isopropyl ether-1-butanol-95% formic acid (30:30:20). The second chromatography was carried out with two solvent systems: isopropyl alcohol-ammonia-0.1 M boric acid (70:10:20) to isolate deoxycytidine, and isopropyl alcohol-concentrated hydrochloric acid-water (85:16:6:18.4) for the isolation of deoxyuridine. When purine ribonucleotides were used as substrate, deoxyadenosine was separated from adenosine, adenine, inosine, hypoxanthine, and xanthine by paper chromatography; deoxyguanosine was separated from guanosine, guanine, xanthosine, and xanthine. The first solvent system for the separation of purine deoxynucleosides used isopropyl alcohol-ammonia-0.1 M boric acid (70:10:20) and the second solvent was distilled water. The appropriate carriers were used for each chromatography. Positions of marker compounds were noted under ultraviolet light and the spots were cut out. The radioactivity present in the deoxy-
Comparison of CTP, CDP, and CMP as substrate for nucleotide reductase

| Substrate  | Specific activity (nmol/mg) |
|------------|-----------------------------|
| CTP        | 0.29                        |
| CDP        | 0.47                        |
| CMP        | 0.23                        |

*All substrates were tested at the 0.5 mM level according to the assay system described in the text.

Fig. 4. Effects of deoxyribonucleoside triphosphates (A) and ribonucleoside triphosphates (B) on CDP reduction; standard assay condition of CDP-"C was used except for addition of effectors and elimination of ATP. •—•, dCTP; X—X, dTTP; ■—■, dATP; ▲—▲, dUTP; ○—○, dGTP; □—□, ATP; △—△, UTP; ○—○, GTP.

Fig. 4. Effects of deoxyribonucleoside triphosphates on CDP reduction; standard assay condition of CDP-"C was used except for addition of effectors and elimination of ATP. •—•, dCTP; X—X, dTTP; ■—■, dATP; ▲—▲, dUTP; ○—○, GTP.

Fig. 5. Effects of deoxyribonucleoside triphosphates on CDP reduction; standard assay condition of CDP-"C was used except for addition of effectors and elimination of ATP. •—•, dCTP; ■—■, dATP; ▲—▲, dUTP; X—X, dTTP.

Fig. 6. Effects of deoxyribonucleoside triphosphates (A) and ribonucleoside triphosphates (B) on UDP reduction; standard assay condition of UDP-"C was used except for addition of effectors and elimination of ATP. •—•, dCTP; ▲—▲, dUTP; X—X, dTTP; ■—■, dATP; ○—○, dGTP; □—□, ATP; △—△, GTP.

RESULTS

The specific activity of ribonucleotide reductase in the spleen following infection with murine leukemia virus is shown in Fig. 1. On the 2nd day after inoculation, the specific activity had risen to over 3 times that of the control. Over the next 3 days, there was a sharp increase in the enzyme level to a peak specific activity approximately 13 times higher than the average for normal spleen. Thereafter, a gradual decline occurred and, by Day 14, the mean specific activity had fallen to about 3 times that of the normal level. The specific activity in the spleen of the phenylhydrazine-treated animals was 1.5 to 3 times that of controls.

The purification of ribonucleotide reductase from leukemic spleen is summarized in Table I. After ammonium sulfate precipitation, the enzyme became very labile, losing 50% of its activity when stored at 4°C and 90% of its activity when kept at room temperature for 40 hours. Attempts to stabilize the enzyme with 2-mercaptoethanol (10⁻⁴ M), dithiothreitol (10⁻⁴ M), ascorbic acid (10⁻⁴ M), Mg²⁺ (10⁻⁴ M), Ca²⁺ (5 × 10⁻² M), or phenylmethyl sulfonyl fluoride were without effect. dCTP and ATP did not stabilize the enzyme for storage or purification. Precipitation of the preparation with alumina C₁₅ or calcium phosphate gel, chromatography on DEAE-cellulose, or further filtration through Sephadex resulted in either no further increase or a loss in specific activity. A loss in activity with dialysis has been reported.
Fig. 7. Effects of deoxyribonucleoside triphosphates (A) and ribonucleoside triphosphates (B) on ADP reduction; standard assay condition of ADP-14C was used except for addition of effectors and elimination of ATP. o---o, dCTP; A---A, dUTP; X---X, dTTP; R---R, dATP; O---O, dGTP; CTP; A---A, UTP; o-0, ATP.

for the ribonucleotide reductase from Yaba Pox virus tumor and some normal monkey tissues (16).

Requirement of Reaction—Fig. 2 shows the effects of magnesium acetate, ferric chloride, dithiothreitol, and ATP on the reaction. The reduction showed a requirement for dithiothreitol and ATP. Higher concentrations of ATP were markedly inhibitory. Slight stimulation by Mg++ was observed. Higher concentrations of Mg++ or Fe+++ ion result in moderate inhibition.

General Properties of Enzyme—The ribonucleotide reductase from murine leukemic spleen was not stimulated by the addition of deoxyadenosyl H2 in a concentration of 10^{-5} M. The optimal pH for the reduction of CDP (in potassium phosphate buffer) occurs between pH 6.8 and pH 7.4, as shown in Fig. 3A. A linear relationship between the concentration of enzyme and the reaction rate was observed (Fig. 3B). When a time study of the reduction of CDP was carried out (Fig. 3C), it was found that the rate decreased after 10 min.

Cytidine Nucleotide Specificity—A comparison of different cytidine nucleotides as substrates is shown in Table II. The highest reduction rate was obtained with CDP. The reaction proceeded at approximately two-thirds maximal rate when CMP or CTP was substituted for CDP. Since phosphatases and kinases were still present in the preparation, it seems likely that some conversion of the tri- and monophosphate to the nucleoside diphosphate may have occurred.

Nucleotide Activation and Inhibition Effects on Reduction of CDP—A comparison of the effects of different nucleoside triphosphates on CDP reduction is shown in Fig. 4. The reduction of CDP was greatly influenced by the presence of different nucleotides. The addition of dCTP and ATP resulted in marked stimulation of the reaction; dTTP and dATP were less effective than dCTP and ATP. It is difficult to show any inhibitory effects in the absence of ATP because of the very low reduction rate. The effects of nucleotides on CDP reduction in the presence of 2 mM ATP are shown in Fig. 5. Low concentrations of dCTP stimulate the reduction of CDP, whereas dTTP, dATP, and larger amounts of dCTP inhibit the reaction.

Reduction of UDP—The effects of nucleotides upon the reduction of UDP are shown in Fig. 6. ATP markedly stimulated the reaction. Slight stimulation by dATP was also noted on UDP reduction. The striking stimulatory effect which dCTP has on CDP reduction is lacking when UDP is the substrate.

Reduction of GDP—The addition of dTTP resulted in a striking increase in GDP reduction, as shown in Fig. 8. Optimal activity was observed at a dTTP concentration of 1 x 10^{-4} M. dUTP and dGTP showed lower stimulatory effects. ATP stimulated this reaction slightly. Although not shown in the figure, in the presence of dTTP, the reduction of GDP was inhibited by dATP to the same extent as the reduction of CDP (Fig. 5).

Similar rates were obtained when the same purified enzyme preparation was tested with CDP, UDP, GDP, or ADP as substrate. In the absence of ATP or other effectors, the barely detectable reaction rates ranged from 0.01 to 0.02 mmole of ribonucleotide reduced per mg of protein per 15 min. With optimal effector concentrations, a 7 to 30-fold increase in reaction rate was observed (Figs. 4 to 7).

No differences were found between the enzymes purified from leukemic spleens, normal spleen, and spleens of control animals injected with phenylhydrazine. ATP and dCTP stimulated CDP reduction. dATP and dGTP stimulated reduction of ADP and GDP. The activities were too low to allow any conclusion concerning inhibition by negative effectors.

**DISCUSSION**

The marked increase in the specific activity of ribonucleotide reductase in a rapidly growing neoplastic tissue is consistent
with the function of the enzyme in providing deoxyribonucleotide precursors for DNA synthesis. The decline in specific activity after Day 6 was unexpected since the spleen continued to increase in size, doubling its weight over the next 4 days. It is conceivable that sufficient amounts of deoxyribonucleotides had accumulated to allow DNA synthesis to continue without a further increase in the enzyme. Repression of further enzyme synthesis by a metabolite offers a possible explanation for the decrease in ribonucleotide reductase activity.

The purification of ribonucleotide reductase from leukemic spleen, like that of the enzyme from Novikoff hepatoma cells, has been complicated by the enzyme's instability during storage or manipulation. Two differences between the spleen enzyme and that from Novikoff hepatoma were noted during the purification procedure. Gel filtration of the spleen enzyme through Sephadex G-25 results in a greater increase in specific activity than does dialysis or gel filtration of the Novikoff hepatoma enzyme. Since there was a 5-fold increase in total activity of the spleen enzyme following this step, it seems probable that a significant quantity of endogenous nucleotides were removed, this would result in less dilution of the radioactivity in the ribonucleotide added to the assay and could account for the increase in specific and total activity. The removal of an inhibitor by Sephadex filtration would offer an alternative explanation for this finding. The second difference noted in the spleen ribonucleotide reductase was that the precipitation at pH 5.0 (18, 22), a useful step in the course of the purification of the enzyme with this substrate. It is possible that the quantitative activity of the spleen enzyme.

The requirements of the enzyme from leukemic spleen are very similar to those of the Novikoff hepatoma (18). There is an absolute requirement for a hydrogen donor, dithiothreitol. Mg++ and Fe+++ ions in low concentrations stimulate and in high concentrations inhibit the reaction. As in the case of the E. coli system, the nucleoside diphosphate is the preferred substrate. In this respect, the enzyme differs markedly from L. leichmannii ribonucleotide reductase, in which the reduction occurs at the triphosphate level (23). The L. leichmannii ribonucleotide reductase, unlike the enzyme from E. coli, shows an absolute requirement for a B12 coenzyme. No stimulation by B12 coenzyme was observed with the mouse spleen ribonucleotide reductase, although the relatively small purification of the enzyme makes this finding of little significance. However, when B12 coenzyme was added to an assay for ribonucleotide reductase in the B12-depleted bone marrow of three patients with pernicious anemia, no stimulation was observed (20). This finding indicates that the bone marrow enzyme does not require deoxyadenosyl B12.

The influence of various nucleotides, which may serve as allosteric effectors in this system, was determined with enzyme from leukemic spleen. The effect of these compounds on the reaction rate closely resembled that observed for the enzyme from E. coli and Novikoff hepatoma and differed from that of the L. leichmannii enzyme. With the present enzyme, there was an almost absolute requirement for ATP in the reduction of CDP and UDP. The ATP-stimulated reaction was markedly inhibited by dATP and dTTP. As has been previously documented for the E. coli and Novikoff hepatoma enzymes by the meticulous studies of Reichard and Moore (7, 8, 24), the effects of dTTP and dATP are dependent upon the concentration of ATP. The two deoxyribonucleotides can therefore function as either inhibitors or activators. dCTP markedly stimulated the reaction in the absence of ATP and also caused a slight stimulation if ATP was present in the reaction. It is worth noting that dCTP acts as an allosteric stimulator on the CDP reduction in the murine leukemia spleen while it inhibits the E. coli tumor enzyme.

As has been observed in the other systems, dTTP stimulates the reduction of the purine nucleotides, and dTTP is the most potent stimulator of GDP reduction in the E. coli, Novikoff hepatoma, and leukemic spleen system. dUPT and dGTP are almost as effective in stimulating the reaction. This relationship is reversed in ADP reduction, in which the greatest stimulation occurs with dGTP, and dTTP stimulation is considerably less than that observed with dGTP. A marked stimulation of ADP reduction by GTP was also noted in this study.

The positive and negative influence of nucleotide effectors in the leukemic spleen ribonucleotide reductase follows the general pattern first described for the E. coli enzyme and even more closely resembles the regulatory controls observed in the Novikoff hepatoma enzyme. Some differences, however, exist between these neoplastic tissues. For example, dCTP which is a weak stimulator of CDP reduction in the Novikoff hepatoma reductase has a marked effect on the spleen enzyme. On the other hand, GTP, which stimulates CDP reduction in the hepatoma system, does not appear to affect the reaction of the spleen enzyme with this substrate. It is possible that the quantitative variation in these effectors may stem from further interconversions or from differences in their rates of degradation by the relatively impure enzyme preparations. The effects of nucleotides on ribonucleotide reductase from murine leukemia spleen are summarized in Table III.

Despite the increase in ribonucleotide reductase activity in the leukemic spleen, no differences in the regulation by nucleotides were found from enzyme purified from normal spleen. The lack of qualitative differences might favor an increase in synthesis de novo of ribonucleotide reductase by the host cells over the coding for a different enzyme by the murine leukemia virus, as an explanation for the elevated enzyme level.

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