COMPLETE DEPENDENCY OF POLY(ADP-RIBOSE) SYNTHESIS ON DNA AND ITS INHIBITION BY ACTINOMYCIN D

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SUMMARY

An enzyme in rat liver chromatin which is capable of polymerizing the ADP-ribose moiety of NAD is dissociated from DNA by the use of CsCl density gradient centrifugation and partially purified by hydroxylapatite and carboxymethyl-cellulose column chromatography. The enzyme which was purified 130-fold showed absolute requirement of DNA. In the presence of DNA, a partial dependency on histone was demonstrated. The enzymic system was inhibited by actinomycin D.

Polyadenosine-diphosphoribose[poly(ADP-Ribose)] has been shown to be synthesized from NAD by an enzymic process in mammalian cell nuclei (1-5). The homopolymer is attached at one end to chromosomal proteins, principally histones (6,7). The biological meaning of poly(ADP-Ribose) or of its binding to chromosomal proteins is not yet fully understood. However, several recent approaches have suggested some correlation between poly(ADP-Ribose) synthesis and regulation of DNA synthesis in mammalian cell. That is, poly(ADP-Ribose) synthesis in nuclei or chromatin diminishes the priming activity of the chromatin in a cell-free DNA synthesizing system, and the activity is restored by removal of the poly(ADP-Ribose)-bound chromosomal proteins from the chromatin (8,9). A decrease in poly(ADP-Ribose) polymerase activity during the stage of DNA synthesis of synchronized cultured cells has been also reported (10). However, only little is known about the properties of the enzyme and the difficulty seems to be partly due to its tight binding to DNA.

Yamada et al. (11) showed that the enzyme can be partially solubilized and purified from rat liver chromatin by DNase treatment. In the present communication a purification method will be presented by which poly(ADP-Ribose) polymerase free of DNA was obtained. The enzyme was purified by CsCl density gradient centrifugation and chromatography on hydroxylapatite and carboxymethyl-cellulose columns. The enzyme thus obtained is completely dependent on DNA and requires histone to exhibit full activity. The reaction will be shown to be inhibited by actinomycin D.

MATERIALS AND METHODS

Materials: Ammonium sulfate (biochemical analysis grade) and CsCl were purchased from Nakarai Chemicals, Kyoto, Japan. Actinomycin D was obtained from Merck Sharp and Dohme. NAD-(adenosine)-^{3}H was prepared after Ueda and Yamamura (12). Histones were prepared by direct extraction of calf thymus nuclei with 0.25 N HCl. The preparation of DNAs is described in the legend to Table 1.

Determinations: Protein was determined after Kihara and Kuno (13). DNA was estimated according to the method of Burton (14), or by measuring its absorbance at 260 nm.

Assay of Reaction: The standard reaction mixture contained 2.5 mμmoles of NAD-(adenosine)-^{3}H (20,000 cpm per mμmoles), 7.5 μmoles of $MgCl_2$, 1.25 μmoles of NaF, 10 μmoles of Tris-Cl buffer (pH 8.0), 440 μmoles of ammonium sulfate and the enzyme preparation in a total volume of 0.25 ml. The mixture was incubated at 25°C for 4 hours and the reaction was stopped by the addition of 5 ml of 10% trichloroacetic acid. The mixture was filtrated on Millipore filter and washed 6 times with 3 ml portions of ice-cold 10% trichloroacetic acid. The radioactivity of the acid-insoluble materials on the dried filter was measured with a liquid scintillation spectrometer. The addition of 1.6 to 1.8 M ammonium sulfate to the reaction mixture inhibited the degradation of the product without affecting the reaction rate (15). Under the described conditions, the reaction was linear for at least five hours.

RESULTS

1) Partial Purification of Enzyme: Liver nuclei of Wistar King strain rats were isolated according to the method of Chauveau et al. (16). The chromatin fraction containing more than 90% of both the enzyme activity and nuclear DNA was extracted from the nuclei and was subjected to CsCl density gradient centrifugation as described in the legend to Fig. 1. The enzyme was completely separated from the bulk of chromosomal nucleic acid with a recovery of 70%. The enzyme preparation (Fr.8 to 15 in Fig.1.) showed no activity unless either DNA or heat-inactivated rat liver chromatin was supplemented to the reaction mixture. The dissociated enzyme was further purified by hydroxylapatite column chromatography. At this stage, the enzyme is purified about 10-fold and contains negligible DNA. The enzyme preparation was used for all further studies unless otherwise stated. The enzyme, obtained with hydroxylapatite column chromatography, was further purified by carboxymethyl-cellulose column chromatography. The final purification was 130-fold with a 22% yield from chromatin-bound enzyme fraction. The enzyme was used for the study of histone requirement of the reaction. Details of the purification procedure will be

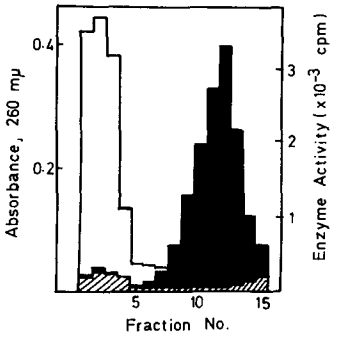

Fig. 1. CsCl density gradient centrifugation of chromatin-bound rat liver poly(ADP-Ribose) polymerase.

Nuclei obtained from 50 g rat liver were washed successively with 30 ml each of Tris-Cl buffer(0.01 M, pH 7.4 and 0.05 M, pH 7.8) and NaCl(0.075 M)-EDTA(0.024 M, pH 8.0) after Ueda et al.(18), and homogenized in 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 5 mM NaF, 30% glycerol (designated as TENG solution pH 8.0 for convenience), containing 1.0 M NaCl, followed by stirring for 2 hours. Then the homogenate was pushed through a hypodermic needle two times and centrifuged at 105,000 g for 1 hour. Clear opalescent supernatant, containing more than 90% of both nuclear DNA and poly(ADP-Ribose) polymerase activity, was adjusted to about 2 mg DNA per ml. CsCl(0.7 g per ml) was added to the chromatin fraction and 4 ml portions of the fraction were laid in 12 ml nitrocellulose tubes. Two ml each CsCl solutions(0.6 g and 0.4 g CsCl per ml of TENG solution pH 8.0) and 4 ml of liquid paraffin were over-laid in the tubes, successively. After centrifugation at 180,000 g for 15 hours the tubes were punctured at the bottom and the samples were fractionated. Upper two-thirds parts were collected and added with more 0.2 g of CsCl per ml and recentrifuged as described above. Centrifugations were performed at -2°C.

The results of the second CsCl density gradient centrifugation are shown in this figure. A 100 μl aliquot of each fraction was added to 2.4 ml of 0.5 M ammonium sulfate and the absorbance at 260 nm was measured. A 5 μl aliquot of each fraction was used for enzyme assay in the standard conditions. White column indicates absorbance at 260 nm. Black and obliquely lined columns indicate enzyme activities in the presence and absence of heat-inactivated rat liver chromatin, respectively, (rat liver chromatin was heated at 50°C for 20 minutes to inactivate poly(ADP-Ribose) polymerase activity completely and used in the experiment).

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described elsewhere. The enzyme could be stored for several weeks in the presense of 30-40% glycerol at -20°C without any appreciable loss of activity (17).

2) DNA-dependency: The partially purified preparation of the enzyme absolutely required DNA for the reaction, and single stranded DNA(phage ØX 174) as well as double stranded DNAs was effective. However, neither yeast tRNA, E. coli rRNA nor heparin was effective in the reaction (Table 1.).

The stimulating effect of DNA on the reaction was also examined with varying amount of rat liver, phage ØX 174 or phage T4 DNA (Fig. 2.). The enzyme activity was saturated with an excess of each DNA (5 to 10 μg per tube). However,

Table 1. DNA requirement of partially purified poly(ADP-Ribose) polymerase.
Enzyme activity was assayed in the standard reaction mixture in the presence of enzyme preparation (5 μg protein), 2 μg calf thymus histone and various materials indicated. Each activity is expressed by percentage of the activity obtained with 10 μg rat liver DNA as 100. Various species of DNA were prepared as follows. Mammalian, E. coli, phage T4 and T6, and phage ØX 174 DNA were prepared by the methods of Kay, Simmons and Dounce(19), Marmur(20), Mandel and Hershey(21), and Sinsheimer(22), respectively. RNAs were donated from Dr. Kuwano(Cancer Res. Inst., Kyushu Univ.).

added material	amount(μg)	% activity of enzyme
no addition	-----	4
rat liver DNA	10	100
calf thymus DNA	10	74
E. coli DNA	10	46
phage T4 DNA	10	27
phage T6 DNA	10	33
phage ØX 174 DNA	10	67
yeast tRNA	20	4
E. coli rRNA	20	4
heparin	20	4

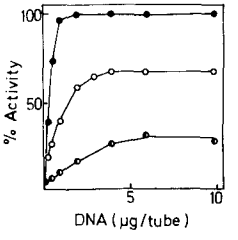

Fig. 2. The effect of various concentrations of DNA on enzyme activity.
The enzyme activity was assayed as described in the legend to Table 1. with either rat liver DNA ●--●, phage ØX 174 DNA o--o, or phage T4 DNA ◐--◐ .

the level of saturation showed some variation among the used DNAs. Saturated enzyme activity of 67 and 30% of that with rat liver DNA was observed with an excess of ØX 174 and T4 DNA, respectively. At low concentration of DNA (0 to 2 μg per tube), the DNAs also showed variation in the ability to stimulate poly(ADP-Ribose) synthesis. With a fixed amount (1 μg per tube) of each rat liver, ØX 174 and T4 DNA, the enzyme activity was 96, 38 and 11% of the maximum activity attained with 10 μg rat liver DNA, respectively.

3) Inhibition by Actinomycin D and Other Dyes: Effect of several dyes, capable of binding to DNA, was tested for poly(ADP-Ribose) synthesis. Actinomycin D, ethidium bromide and proflavin, thus examined, were observed to inhibit the reaction by 90, 40 and 40%, respectively, at the final concentration of 8 μg per ml. The inhibitory effect of varying concentration of actinomycin D is shown in Fig. 3.

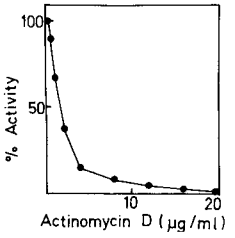

Fig. 3. Inhibition of polyADPR synthesis by actinomycin D.
The activity was assayed under the standard conditions with 2 μg each of rat liver DNA and calf thymus histone in the presence of various concentrations of actinomycin D.

Pre-treatment of DNA with actinomycin D remarkably diminished the activity of the DNA in the reconstructed enzyme system. Addition of non-treated DNA to the reaction mixture did not support the enzyme activity at all after the enzyme had interacted with the actinomycin D-bound DNA in the reaction mixture. However, the actinomycin D-bound DNA did not show any inhibitory effect if the enzyme had interacted with non-treated DNA, prior to its addition.

4) Histone-dependency: The requirement of histones in the reaction was determined with the use of the final purified enzyme preparation (0.2 μg protein) and with 2 μg rat liver DNA in the standard reaction mixture. The reaction rate was stimulated to 230% of the control value with the addition of 2 ug calf thymus histones. The use of bovine serum albumin in place of histones showed no stimulating effect. Even when histones were added, the enzyme activity was nil when DNA was omitted.

5) Nature of Reaction Product: The reaction product synthesized with the reconstructed enzyme system is resistent to pronase, RNase and DNase, is hydrolyzed by snake venom phosphodiesterase and is relatively acid-labile and alkali-stable as described by other investigators (2,5).

DISCUSSION

Poly(ADP-Ribose) synthesis has been suggested to be dependent on DNA by Nishizuka et al. (2, 23). In these reports, we showed that prior treatment of

chromatin with DNase results in the formation of polymers of decreasing chain length. With the use of partially purified preparation of the polymerase free of DNA, the present study established that DNA is absolutely required for poly-(ADP-Ribose) synthesis. All of double and single stranded DNAs obtained from phages, bacteria and eukaryotes, thus far examined, were effective in the reaction. However, none of other polyanions, such as RNA and heparin, supported the polymerization reaction. When the effect of DNA was examined with varying amount of DNA, the difference in both of the maximum stimulation and stimulating efficiency was observed among DNA species (Fig.2).

The inhibitory effects of actinomycin D and other dyes also suggest that a specific entity of DNA may be required for the reaction, because the effect seems to be dependent on the binding of the dyes to DNA. Pre-treatment of DNA with actinomycin D remarkably diminished the activity of the DNA in the reconstructed enzyme system. It is also suggested that actinomycin D-bound DNA may be able to bind the enzyme in spite of its inability of stimulating the reaction, since the addition of non-treated DNA to the reaction mixture did not support the enzyme activity after the interaction of the enzyme with actinomycin D-bound DNA.

All of these results described above, lead us to presume some structure in DNA may be used for poly(ADP-Ribose) formation. An interesting problem is to see whether or not one of specific base sequences in DNA is a prerequisite for the reaction to occur. However, our recent results indicated that all of synthetic deoxyribonucleotide polymers and DNA-RNA hybrid, such as poly[d(A-T)], poly d(G).poly d(C), and poly d(T).poly r(A), were effective when they were used in place of natural DNA in the reconstructed enzyme system, except that single stranded poly d(T) showed no stimulating effect by itself but strongly inhibited the reaction when added to the reaction mixture supplemented with rat liver DNA (24). The obserbations by Yamada et al.(11) also indicated that poly[d(A-T)] and poly d(G).poly d(C) were effective in their reconstructed enzyme system. It seems, therefore, unlikely that one of specific base sequences in DNA is a prerequisite for the reaction to occur, although the reason why single stranded polyd(T) does not stimulate but inhibit the reaction, is not yet clarified.

Poly(ADP-Ribose) synthesis was stimulated about 2-fold by the addition of histones in the presence of DNA. An absolute requirement for exogenous histones was not demonstrated in our reconstructed enzyme system. However, it is possible that the final purified enzyme preparation contains some histones.

The more exact understanding for the role of DNA and histones in poly(ADP-Ribose) synthesis will be the subject of a forthcoming communication.

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REFERENCES

1. Chambon, P., Weil, J. D., and Mandel, P., Biochem. Biophys. Res. Commun., 11, 39 (1963).
2. Nishizuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O., J. Biol. Chem., 242, 3164 (1967).
3. Reeder, R. H., Ueda, K., Honjo, T., Nishizuka, Y., and Hayaishi, O., J. Biol. Chem., 242, 3172 (1967).
4. Fujimura, S., Hasegawa, S., Shimizu, Y., and Sugimura, T., Biochim. Biophys. Acta, 145, 247 (1967).
5. Hasegawa, S., Fujimura, S., Shimizu, Y., and Sugimura, T., Biochim. Biophys. Acta, 149, 369 (1967).
6. Nishizuka, Y., Ueda, K., Honjo, T., and Hayaishi, O., J. Biol. Chem. 243, 3765 (1968)
7. Otake, H., Miwa, M., Fujimura, S., and Sugimura, T., J. Biochem. 65, 145 (1969)
8. Burzio, L., and Koide, S. S., Biochem. Biophys. Res. Commun., 40, 1013 (1970)
9. Burzio, L., and Koide, S. S., Biochem. Biophys. Res. Commun., 42, 1185 (1971).
10. Smulson, M., Henriksen, O., and Rideau, C., Biochem. Biophys. Res. Commun., 43, 1266 (1971).
11. Yamada, M., Miwa, M., and Sugimura, T., Arch. Biochem. Biophys., 146, 579 (1971).
12. Ueda, K., and Yamamura, Y., "Methods in Enzymology", Academic Press, New York and London, 18 (part B), 60-66 (1971).
13. Kihara, H. K., and Kuno, H., Anal. Biochem., 24, 96 (1968).
14. Burton, K., Biochem. J., 62, 315 (1956).
15. Nishizuka, Y., Ueda, K., Nakazawa, K., Reeder, R. H., Honjo, T., and Hayaishi, O., J. Vitaminol., 14, 143 (1968).
16. Chauveau, J., Moulè, Y., and Rouiller, Ch., Exp. Cell Res., 11, 317 (1956).
17. Yamada, M., Miwa, M., and Sugimura, T., J. Jap. Biochem. Soc., 42, 477 (1970).
18. Ueda, K., Reeder, R. H., Honjo, T., Nishizuka, Y., and Hayaishi, O., Biochem. Biophys. Res. Commun., 31, 379 (1968).
19. Kay, E. R. M., Simmons, N. S., and Dounce, A. L., J. Am. Chem. Soc., 74, 1724 (1952).
20. Marmur, J., J. Mol. Biol., 3, 208 (1961).
21. Mandel, J. D., and Hershey, A. D., Anal. Biochem., 1, 66 (1960).
22. Sinsheimer, R. L., "Procedures in Nucleic Acid Research", Harper and Row, New York and London, 569 P. (1966).
23. Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., and Hayaishi, O., Cold Spr. Harb. Symp. Quant. Biol., 34, 781 (1969).
24. Yoshihara, K., manuscript in preparation.