POLY(ADP-RIBOSE) GLYCOHYDROLASE IN MOUSE FIBROBLAST CELLS (LS CELLS)

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Received 30 July 1973

1. Introduction

The nuclei of eukaryotic cells contain an enzyme which converts NAD into poly(ADP-ribose)* with the elimination of nicotinamide [1–4]. This enzyme, poly(ADP-ribose) polymerase, is chromatin bound and dependent upon DNA for activity [1, 5, 6]. The first ADP-ribose moiety from NAD is covalently linked to chromosomal proteins and then additional units are successively added to these protein bound poly(ADP-ribose) units [6, 7].

Two hydrolytic enzymes have been implicated in the degradation of this polymer. A phosphodiesterase splits the pyrophosphate bonds [8] and a poly(ADP-ribose) glycohydrolase splits the glycosidic ribosyl (1′–2′) ribose linkage [9, 10]. The ribosyl (1′–2′) ribose linkage is the characteristic bond formed between successive ADP-ribose units in the polymer. This poly(ADP-ribose) glycohydrolase has been shown to be associated with the chromatin fraction of rat liver nuclei [11]. It has also been partially purified from the nuclear soluble fraction of rat liver [10]. Until now, the glycohydrolase has been identified only in rat liver and rat thymus cells [9–11].

In this paper we report the presence of poly(ADP-ribose) glycohydrolase in nuclei isolated from mouse fibroblast cells (LS cells) grown in suspension culture.

A new simple method is described for identifying the products resulting from the degradation of poly(ADP-ribose). The undigested polymer is precipitated with 66% ethanol (v/v) and the degradation products ADP-ribose, phosphoribosyl-AMP remain in the ethanol supernatant. After evaporation of the supernatant the nucleotides are separated from NAD by step-wise elution on Dowex-1-formate. The 3 M formic acid eluate from the Dowex-1-formate column is digested with alkaline phosphatase or with nucleotide pyrophosphatase. The products are analysed on PEI-cellulose plates both before and after enzymic digestion. This discriminates between ADP-ribose and phosphoribosyl-AMP.

* *Abbreviation:* poly (ADP-ribose) – poly adenosine diphosphoribose.

2. Materials and methods

2.1. *Cell line*

Mouse fibroblast-cells, clone LS929, a gift of Dr. John Paul (Glasgow) were grown in suspension culture in Minimal Essential Medium (Biocult Labs) supplemented with 5% bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin and 0.2% $NaHCO_3$.

2.2. *Nuclei preparation*

Nuclei were prepared by a modification of the method of Traub [12]. LS cells (about 56×10^6) were harvested and counted in a Coulter counter (Model D), centrifuged at 1000 *g* for 10 min, resuspended in distilled water (at a concentration of about 8×10^6 cells/ml) and left to stand for 10 min at 4°C. The suspension was then gently disrupted by several strokes of a hand homogeniser. Two ml of this preparation were then centrifuged through a two layer sucrose solution in cellulose nitrate tubes. The lower layer consisted of 1 ml of 2 M sucrose and the upper layer of 2 ml of 0.047% (w/v) sodium dodecyl sulphate, 0.5% (w/v) Tween 20 and 0.25% (w/v) sodium deoxycholate in 0.25 M sucrose. The tubes were spun in an SW39L rotor in a Beckman L-2 ultracentrifuge first

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Table 1
Analysis of alcohol supernatants from time course of degradation.

	Counts per minute					
	+ Nicotinamide			– Nicotinamide		
Spot on TLC sheet	Undigested	Alkaline phosphatase	Nucleotide pyrophosphatase	Undigested	Alkaline phosphatase	Nucleotide pyrophosphatase
ADPR	2192	2567	164	5062	5965	233
AMP	288	312	3066	244	676	6168

The remaining reaction mixtures left at 20.5 hr from the time courses of degradation in the presence and absence of nicotinamide (fig. 1) were centrifuged and the supernatants run on Dowex-1-formate columns; the 3 M formic acid eluates digested with alkaline phosphatase or with nucleotide pyrophosphatase and these digests run on PEI-cellulose sheets as described in Materials and methods.

at 7 500 *g* for 15 min and then at 100 000 *g* for 45 min. The nuclear pellet from the bottom of the tube was resuspended in distilled water at a concentration of about 10^7 nuclei/ml. The nuclei do not burst in water at 4°C.

2.3. *Poly(ADP-ribose) polymerase assay*

The method is essentially that of Nishizuka et al. [2]. The reaction mixture contained 3.34 mM KF, 3.34 mM mercaptoethanol, 50 mM KCl, 83.5 mM Tris–HCl pH 8.5, 2.0 mM $MgCl_2$ and 1.0 to 1.3×10^6 nuclei/ml. The reaction was started by the addition of [^{3}H]NAD (22.2 Ci/mM, 160 Ci/ml) to give a final NAD concentration of 1.22×10^{-7} M. At various times 0.3 ml aliquots were removed and the reaction terminated by the addition of 5 ml 5% TCA. The samples were left on ice for at least one hour and the acid-precipitable radioactivity measured, after filtration on glass fibre discs, by liquid scintillation counting.

2.4. *Degradation of poly(ADP-ribose)*

Poly(ADP-ribose) synthesis and degradation occur simultaneously if both enzymes are present. Thus, we allowed the synthesis of polymer to occur and then examined the solution for evidence of simultaneous degradation. Degradation of the polymer by the glycohydrolase would give rise to ADP-ribose; this could also arise directly from NAD. Control experiments showed that NAD hydrolysis to ADP-ribose did not occur in these nuclei (table 1).

The synthesis of polymer was carried out as in section 2.3, except that the reaction was stopped by the addition of ethanol to a final concentration of 66%. The samples, after being kept at 0°C for at least 24 hr, were centrifuged at 1000 *g* for 10 min to remove insoluble material. The alcohol was removed from the supernatant with a stream of dry air, and the resulting solution quantitatively applied to a Dowex-1-formate column (4 × 60 mm). The column was then successively eluted with 20 ml of 0.03 M formic acid, 20 ml of 0.3 M formic acid and finally 20 ml of 3.0 M formic acid. The 3.0 M formic acid eluate was collected and freeze dried. This sample was then resuspended in 0.5 ml distilled water.

2.5. *Digestion of sample*

Samples of the 3 M formic acid eluate were digested with alkaline phosphatase (*E. coli* 280 units/ml, Sigma) or with nucleotide pyrophosphatase (snake venom 28 units/ml, Sigma). A 20 μl sample of the freeze-dried preparation was reacted with 10 μl 0.2 M Tris–HCl pH 7.4 and 10 μl of the enzyme mixture (for alkaline phosphatase: 1 ml 0.2 M Tris–HCl, pH 7.4, 10 mM Mg^{2+} + 10 μl alkaline phosphatase and for nucleotide pyrophosphatase – 100 μl 0.2 M Tris–HCl pH 7.4, 10 mM Mg^{2+} + 10 μl nucleotide pyrophosphatase) on a sheet of polythene for 10–15 min at room temperature.

Possible products from poly(ADP-ribose) degradation which would elute in the 3 M formic acid wash are ADP-ribose and 2′(5″-phosphoribosyl)-5′-AMP. Alkaline phosphatase (EC 3.1.3.1) will digest phosphoribosyl-AMP producing ribosyl adenosine while nucleotide pyrophosphatase (EC 3.6.1.9) will digest ADP-

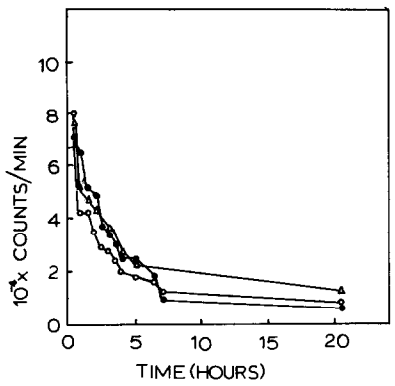

Fig. 1. Degradation of poly(ADP-ribose) by isolated nuclei. 3 ml of nuclei (about 39 X 10^6) were incubated with [^{3}H]NAD (final volume 9 ml) at 25°C for 30 min as described in Materials and methods. Then 0.5 ml 4 M nicotinamide (○—○—○); 0.5 ml water (●—●—●) or 0.5 ml 100 mM NAD (△—△—△) was added. At various times a 0.3 ml sample was taken and the acid-precipitable radioactivity determined as described in Materials and methods.

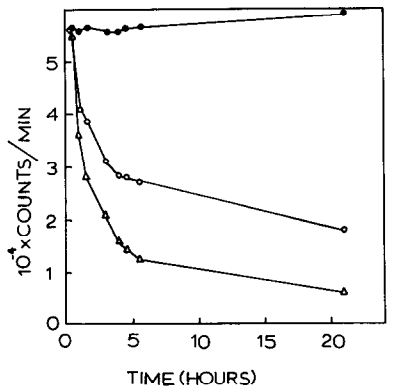

Fig. 2. Inhibition of poly(ADP-ribose) degradation. 1.5 ml of nuclei (about 20 X 10^6) were incubated with [^{3}H]NAD (final volume 4.5 ml) at 25°C for 30 min as described in Materials and methods and then 0.5 ml 4 M nicotinamide was added to each sample to stop polymer synthesis in all tubes. 0.5 ml 100 mM cyclic AMP (○—○—○), 0.5 ml 5 M $(NH_4)_2SO_4$ (●—●—●) or 0.5 ml H_2O (△—△—△) was added to different tubes. At various times a 0.3 ml sample was taken and the TCA-precipitable radioactivity determined as described in Materials and methods.

ribose producing AMP and ribose-1-phosphate. Alkaline phosphatase will not digest ADP-ribose and nucleotide pyrophosphatase will not digest phosphoribosyl AMP. Both enzymes were shown to be free of contaminating enzymes which might have affected the hydrolysis of the product in the 3 M formic acid eluate. Alkaline phosphatase is free of any contaminating phosphodiesterase activity as seen by its inability to release any [^{3}H]AMP from [^{3}H]NAD. Nucleotide pyrophosphatase is free of any contamination by alkaline phosphatase as seen by its inability to release any $^{32}P_i$ from [^{32}P]AMP. Thus PEI-cellulose chromatography of the digest will show whether ADP-ribose or phosphoribosyl-AMP is the degradation product of poly(ADP-ribose) because one can readily separate ribosyl adenosine from phosphoribosyl-AMP and ADP-ribose from AMP.

2.6. *PEI-cellulose thin layer chromatography*

Samples were analysed before and after enzymic hydrolysis. The method is essentially that of Randerath et al. [13]. The digested sample was taken up in a capillary tube from the polythene sheet and spotted on to the PEI-cellulose sheet (J.T. Baker Chemical Co., Phillipsburg, N.J. 08865, U.S.A.) together with the appropriate markers (20 nmoles AMP and 20 nmoles ADP-ribose) as a 1 cm band along the origin. The sheet was developed in 1 M acetic acid until the solvent front had run 2 cm and then in 0.9 M acetic acid–0.3 M LiCl until the solvent front had run another 15 cm. The sheets were then dried and observed under short wavelength ultraviolet light and the AMP, ADP-ribose and ribosyl-adenosine spots were cut out, placed in scintillation fluid in vials and the samples counted in a Packard Tri Carb liquid scintillation counter. The R_f values for ADP-ribose, AMP and ribosyl-adenosine in this solvent system are 0.30, 0.56, and 0.95 respectively.

3. Results and discussion

LS cell nuclei show a loss of poly(ADP-ribose) as indicated by a decrease in acid-insoluble radioactivity with time (fig. 1). This loss of polymer is inhibited completely by 0.5 M $(NH_4)_2SO_4$ or partially by 10 mM cAMP (fig. 2). $(NH_4)_2SO_4$ and cAMP inhibit, to different degrees, the disappearance of poly(ADP-ribose) in rat liver chromatin [14]. The enzyme responsible for the degradation of polymer in rat liver was subsequently shown to be a poly(ADP-ribose) glycohydrolase [11]. The inhibitory effect of $(NH_4)_2SO_4$

and cAMP is also seen on the soluble poly(ADP-ribose) glycohydrolase from rat liver [10]. These compounds do not inhibit rat liver phosphodiesterase. Inhibition of the loss of acid insoluble material formed by poly(ADP-ribose) polymerase from several rat and rabbit organs by $(NH_4)_2SO_4$ and cAMP has been used as evidence that there is poly(ADP-ribose) glycohydrolase activity in these systems [15].

The alternative to poly(ADP-ribose) glycohydrolase hydrolysis of poly(ADP-ribose) is breakdown of polymer by phosphodiesterase activity to give AMP and phosphoribosyl-AMP [8]. To show that the loss of acid-insoluble material was not due to phosphodiesterase activity, LS cell nuclei were incubated with either Ca(bis)*p*-nitrophenyl phosphate, *p*-nitrophenyl-5-thymidine phosphate or *p*-nitrophenyl-3-thymidine phosphate, under conditions used for the poly(ADP-ribose) polymerase assay as described under Materials and methods. Over a period of 12 hr no nitrophenol was produced from any of these substrates.

The decay of poly(ADP-ribose) in LS cell nuclei gives rise to ADP-ribose (table 1), the specific product of the enzyme poly(ADP-ribose) glycohydrolase. That the product of poly(ADP-ribose) hydrolysis is indeed ADP-ribose was shown unequivocably by the fact that nucleotide pyrophosphatase digests it but alkaline phosphatase does not (table 1). The latter enzyme would however have digested phosphoribosyl-AMP had it been formed from the polymer by phosphodiesterase activity.

The ADP-ribose might have arisen from the hydrolysis of NAD by NADase or by non-specific glycohydrolase activity. The absence of these activities was shown by incubating [^{14}C]NAD and nuclei together in the presence of 20 mM thymidine when poly(ADP-ribose) polymerase is completely inhibited [16–18]. Neither ADP-ribose nor AMP are detectable under these conditions. These products are not formed in the absence of polymer synthesis; in this system they only arise from polymer degradation. Thymidine was shown not to inhibit NADase from calf spleen or pig brain (Boehringer, Mannheim).

There is more ADP-ribose produced in the absence of nicotinamide than in its presence (table 1). This is because in the presence of nicotinamide, a competitive inhibitor of poly(ADP-ribose) polymerase [18–20], only degradation of polymer occurs whereas in the absence of nicotinamide both synthesis and degradation of polymer occur.

Thus, as in the rat liver system, nuclei isolated from LS cells have a poly(ADP-ribose) glycohydrolase activity. The interrelation between this enzyme and poly(ADP-ribose) polymerase is, as yet, unknown and, even more important, the physiological role of these two enzymes has yet to be elucidated.

Acknowledgements

This work was supported by the Science Research Council. P.R.S. holds a Research Studentship from the Science Research Council.

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