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Catalytic activities of synthetic octadeoxyribonucleotides as coenzymes of poly(ADP-ribose) polymerase and the identification of a new enzyme inhibitory site

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The catalytic activity of highly purified poly(ADP-ribose) polymerase was determined at constant NAD^+ concentration and varying concentrations of sDNA or synthetic octadeoxyribonucleotides of differing composition. The coenzymic activities of deoxyribonucleotides were compared in two ways: (i) graphic presentation of the activation of poly(ADP-ribose) polymerase in the presence of a large concentration range of deoxyribonucleotides and (ii) by calculating k_D values for the deoxyribonucleotides. As determined by method i, auto-mono-ADP-ribosylation of the enzyme protein at 25 nM NAD^+ was maximally activated at 1:1 octamer/enzyme molar ratios by the octadeoxyribonucleotide derived from the regulatory region of SV40 DNA (duplex C). At a 0.4:1 sDNA/enzyme ratio, sDNA was the most active coenzyme for mono-ADP-ribosylation. At 200 μM NAD^+, resulting in polymer synthesis and with histones as secondary polymer acceptors, duplex C was the most active coenzyme, and the octamer containing the steroid hormone receptor binding consensus sequence of DNA was a close second, whereas sDNA exhibited an anomalous biphasic kinetics. sDNA was effective on mono- ADP-ribosylation at a concentration 150–200 -times lower than on polymer formation. When comparison of deoxyribonucleotides was based on method ii (k_D values), by far the most efficiently binding coenzyme for both mono and polymer synthesis was sDNA, followed by duplex C, with $(dA\text{-}dT)_8$ exhibiting the weakest binding. The synthetic molecule 6-amino-1,2-benzopyrone (6-aminocoumarin) competitively inhibited the coenzymic function of synthetic octadeoxyribonucleotides at constant concentration of NAD^+, identifying a new inhibitory site of poly(ADP-ribose) polymerase.

Octadeoxyribonucleotide; Poly(ADP-ribose) polymerase; 6-Amino-1,2-benzopyrone site

1. INTRODUCTION

Poly(ADP-ribose) polymerase (EC 2.4.99) is a specific DNA-binding nuclear protein of eukaryotes that catalyzes the polymerization of ADP-ribose derived from NAD^+ [1,2]. The polymerization is initiated by auto-mono-ADP-ribosylation of the enzyme protein followed by ADP-ribose transfer to other acceptor proteins and subsequent elongation [3]. The enzymatic process is obligatorily dependent on DNA, and an enzyme-associated DNA defined as sDNA (cf. [4,11]) can be isolated that is more efficient as a coenzyme than crude thymus DNA [5,6]. The exact catalytic mechanism which makes DNA necessary for poly(ADP-ribose) synthesis is not known but there are indications that one of its roles may be involved in ADP-ribose chain elongation [7]. It was reported that introduction of enzymatic cleavage sites into double-stranded DNA coincides with increased rates of poly(ADP-ribose) synthesis [8,9] and a frequently quoted hypothesis developed correlating DNA damage with an in-

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creased rate of poly ADP-ribosylations (cf. [1,2]). Some doubts of the validity of this hypothesis emerged recently since only very few enzyme molecules are located by electron microscopy at the ends of sDNA [10,11]. Furthermore, the existence of highly fragmented DNA does not inevitably coincide with high rates of poly ADP-ribosylation [12]. It was recently concluded on the basis of experiments with synthetic oligo(dA-dT) that maximal activation of poly(ADP-ribose) polymerase in the nucleus may be catalyzed by 'short DNA pieces' not detectable by methods that are suitable for the assay of DNA fragmentation [13].

We have observed that hormonal action in vivo and in cell cultures coincides with a decrease in poly ADP-ribosylation without a diminution of enzyme content [14]. Since a specific interaction between hormone-receptor complexes and certain DNA sequences is well known (cf. [14]), we assumed that the observed correlation between hormone action and decreased rates of poly ADP-ribosylation may suggest altered binding sites for the enzyme on DNA sequences (or structures) that are less efficient coenzymes for poly(ADP-ribose) polymerase than the DNA sites prior to hormone action [14]. This mechanism is consistent with the hormone receptor binding-induced change in chromatin structure (cf. [14]). If this assumption is correct, one may anticipate that oligodeoxyribonucleotide sequences of varying composition in DNA may be significant in determining the coenzymic effectivity of DNA. The present report is concerned with the experimental testing of this hypothesis. We also demonstrate here that application of synthetic oligodeoxyribonucleotides, which are chemically defined coenzymes of poly(ADP-ribose) polymerase, leads to the identification of a novel, DNA-related enzyme-inhibitory site. Drugs which inhibit tumorigenesis [15–17] bind to this DNA-related site.

2. MATERIALS AND METHODS

Two of the synthetic oligodeoxyribonucleotides were based on the $AGA^{A}_{T}CAG^{A}_{T}$ consensus oligodeoxyribonucleotide sequence identified by Yamamoto [18] to be required for hormone-dependent transcriptional enhancement in vivo. Another model was an octadeoxyribonucleotide identified in the regulatory region (203 SphI) of SV40 DNA [19]. The three octadeoxyribonucleotides were synthesized by the solid-phase method [20], and their structures are as follows:

Duplex A: 5′-A-G-A-T-C-A-G-T-3′ ;
3′-T-C-T-A-G-T-C-A-5′

Duplex B: 5′-A-G-A-A-C-A-G-A-3′ ;
3′-T-C-T-T-G-T-C-T-5′

Duplex C: 5′-G-C-A-T-G-C-A-T-3′ ;
3′-C-G-T-A-C-G-T-A-5′

Duplex B differs from A only in bases 4 and 8 replacing T by A.

Each preparation of single-strand octadeoxyribonucleotide of specific sequence was purified by anion-exchange HPLC with a potassium phosphate elution gradient described in [21] wherein the octamer represented the major elution peak. The octamer was then desalted by absorbing it on a Waters reversed-phase Sep-Pak cartridge, washing with water, and desorbing with 60% methanol. After removing methanol by rotary evaporation and freeze-drying, preparations were checked for purity by gel electrophoresis and quantitated by UV spectrometry. Double-strand species were prepared by combining equimolar amounts of complementary single strands in a buffer consisting of 0.05 M NaCl, 6.6 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$ and 1.0 mM dithiothreitol, followed by heating at 100°C for 3 min, and then annealing with a slow cool-down to room temperature. In the same manner the duplex $(dA\text{-}dT)_8$ was prepared from the component single strands, obtained from Pharmacia and purified by HPLC [21]. 6-Amino-1,2-benzopyrone [22] was prepared by the spontaneous reduction of 6-nitrocoumarin (Aldrich no.S42722-5) by iron powder in acetic acid, followed by filtration, rotary evaporation of acetic acid, extraction into ether and crystallization from ethanol (m.p. 166–169°C). Poly(ADP-ribose) polymerase (EC 2.4.99) of 95% homogeneity was isolated as in [6] from calf thymus. Coenzymic DNA (sDNA) was separated from the polymerase protein by hydrox-

yapatite chromatography with a phosphate gradient [6] and the DNA was freed from protein by repeated phenol extraction and alcohol precipitation. This DNA species (sDNA) was a mixture of double strands ranging between 0.5 and 3.5 kb. Enzymatic mono-ADP-ribosylation of the poly(ADP-ribose) polymerase protein was carried out as reported [3] and polymer formation on the enzyme and histones as described in the legend to fig.2.

3. RESULTS AND DISCUSSION

Conventional enzyme kinetic analyses of reactions catalyzed by poly(ADP-ribose) polymerase are frough with uncertainties because only the initial phase of mono-ADP-ribosylation [3] at very low concentrations of NAD^+ approximates Michaelis-Menten kinetics [23]. At saturating concentrations of NAD^+, the rate of polymer formation, which follows a Poisson distribution, becomes important (cf. [23]), therefore the relationship between overall velocity and NAD^+ concentration cannot be described by a simple rate equation. Because of these complications, we compared the catalytic efficiencies of deoxyribonucleotides by two methods. The first consisted of correlating enzymatic activity with the weight (concentration) of deoxyribonucleotides varying over a very large range, over almost 5 orders of magnitude. This large range was necessary because in intact cells 1 mol of enzyme corresponds to 2–500 kb DNA (cf. [24]), therefore catalytic effects at low and high DNA/enzyme ratios could have biological importance. The second method was the calculation of binding constants (k_D) of the octadeoxyribonucleotides and sDNA to the enzyme (table 1).

The coenzymic activities of sDNA, $(dA\text{-}dT)_8$ and octamer duplexes A, B and C were determined on the auto-modification of a constant concentration of poly(ADP-ribose) polymerase (8 pmol/test) under two experimental conditions resulting in mono and poly ADP-ribosylations [3]. In fig.1, auto-mono-ADP-ribosylation of the enzyme was assayed at a constant substrate concentration of 25 nM NAD^+ as ADP-ribose donor [3]. The abscissa shows the log of the concentration of sDNA or octamer duplexes (ng/0.1 ml test) over 5 orders of magnitude. It should be noted that

Table 1

Binding of deoxyribonucleotides to poly(ADP-ribose) polymerase

Deoxyribonucleotide species	Nature of enzymatic assay	
	Mono ADP-ribosylation[a]	Poly ADP-ribosylation[b]
Duplex A	2×10^{-7}	1.4×10^{-7}
Duplex B	2×10^{-7}	1.4×10^{-7}
Duplex C	6×10^{-8}	7×10^{-8}
$(dA\text{-}dT)_8$	8×10^{-7}	4×10^{-7}
sDNA	1×10^{-9}	1.5×10^{-8}

[a] 25 nM NAD^+
[b] 250 μM NAD^+

Results are given as k_D, determined by enzyme kinetics. Each value is an average of 3 determinations (± 10% variation); k_D values were calculated from enzymatic tests (figs 1,2) where deoxyribonucleotides were varied over a concentration range of 10^4

relatively small apparent differences in the saturation curves of deoxyribonucleotides actually correspond to significant differences because of the logarithmic abscissa. The three arrows on top (in figs 1,2) indicate the ratios of mol octamers per mol enzyme in the system. Since the average molecular mass of sDNA was 1.3×10^6, the mol sDNA/enzyme ratio at the first arrow was 0.004:1, at the second 0.04:1 and at the third 0.4:1. At or below an octamer duplex/enzyme ratio of 1:1, duplex C was the most effective coenzyme and $(dA\text{-}dT)_8$ the poorest on the initial rates of auto-mono-ADP-ribosylation of the enzyme. At a large excess of octamer duplexes (ratio 100:1) which may simulate cellular conditions [24] duplex B and $(dA\text{-}dT)_8$ were similar. The effect of sDNA on mono-ADP-ribosylation was biphasic. Below an sDNA/enzyme ratio of 0.004:1 (first arrow) sDNA was hardly effective, whereas increasing the concentration of sDNA to reach an sDNA/enzyme ratio of 0.04:1 (second arrow), the coenzymic effect of sDNA was maximal. A structural contribution of the much larger sDNA species, as compared to the synthetic duplexes, apparently introduces macromolecular effects of sDNA on the enzyme which cannot occur with small DNA analogs. Poly ADP-ribosylation at 200 μM NAD^+ as substrate with an excess of whole thymus

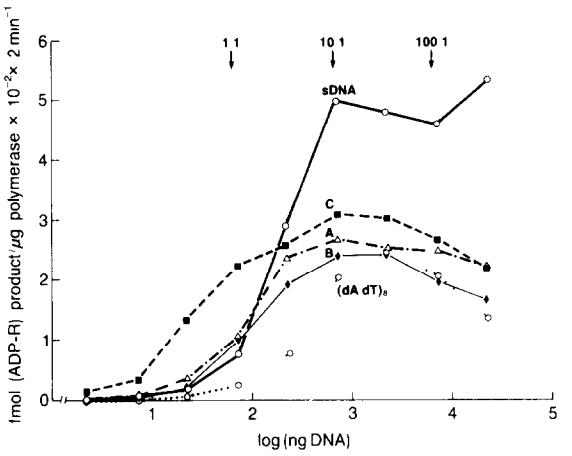

Fig.1. Effect of sDNA and various octadeoxyribonucleotides on initiation activity of poly(ADP-ribose) polymerase. NAD^+ concentration was 25 nM and V_{init} = rates in first 2 min at 24°C. A, B, C denote duplex A, duplex B and duplex C, respectively.

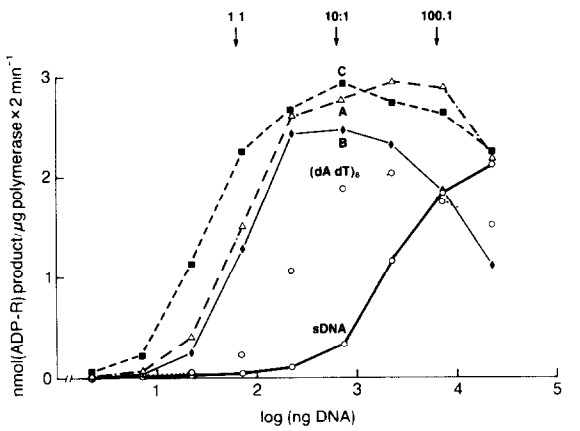

Fig.2. Effect of various concentrations of sDNA and octadeoxyribonucleotides on rates of polymer formation by poly(ADP-ribose) polymerase. NAD^+ concentration was 200 μM and 10 μg whole thymus histones were added per 0.10 ml incubation mixture.

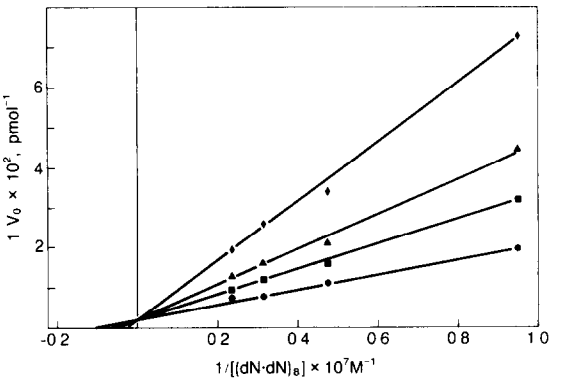

Fig.3. The effect of 6-amino-1,2-benzopyrone on the initial velocity of auto-mono-ADP-ribosylation of the enzyme as a function of varying concentration of octadeoxyribonucleotide. No inhibitor present (●), 20 μM (■), 40 μM (▲) and 80 μM (▼) 6-amino-1,2-benzopyrone. NAD^+ concentration was constant (= 25 nM). The K_i for 6-amino-1,2-benzopyrone is approx. 28 μM, and k_D, based on a 10-fold concentration range for the octadeoxyribonucleotide, is between 0.3 and 0.8 μM. $(dN \cdot dN)_8$ denotes duplex A and its structure is specified in the text. Data points in figs 1–3 represent an average of 3 determinations each.

histones as additional poly(ADP-ribose) acceptors, probably simulating conditions prevailing in chromatin, yielded a less complicated kinetics (fig.2). In this system, on a weight basis, octameric duplex C is the best synthetic coenzyme. Octamer duplex B, which is only slightly different from A, was inhibitory above 4 logs, whereas the activation curve of sDNA remained sigmoidal. The exact reasons for the inhibitory effect at high concentrations of duplex B are unknown, but this inhibition is apparently related to the composition of the duplex. In the cell nucleus, the concentration of DNA is far greater than that of the polymerase [24], therefore the observed inhibition in the in vitro model (fig.2) may have biological relevance and it is possible that certain DNA sequences could be less effective coenzymes.

It may be argued on the basis of previous theories [8,9] that equivalent short pieces of DNA analogs representing many more 'activating termini' than sDNA should increase the catalytic reaction of poly ADP-ribosylation regardless of their base composition. This is not supported by results which show that octamer duplex C is significantly more effective than the others, especially $(dA\text{-}dT)_8$. Furthermore, there is a concentration difference of 2 log units in the activating concentration range of sDNA acting on mono as compared to poly ADP-ribosylation, an effect that is unlikely to be related to DNA fragmentation. When, instead of recording enzyme activities in the

presence of widely varying concentration of deoxyribonucleotides, k_D values are calculated for each macromolecule, results as summarized in table 1 are obtained. Clearly sDNA exhibits the highest affinity, followed by octamer duplex C, and the A and B (being equal) and finally $(dA\text{-}dT)_8$ in decreasing order. The apparent differences in k_D as determined under conditions of mono or poly ADP-ribosylations are probably due to differing kinetics of the two processes [23]. It may be assumed that certain base sequences present in sDNA, being effective coenzymes like duplex C, are much more efficient catalysts when they are a part of the macromolecular DNA structure. We do not know what the 'active' base sequences in sDNA are, but the present results support a significant structural contribution of sDNA, as has been proposed earlier [10,11]. On the other hand, a catalytic activation of poly(ADP-ribose) polymerase by the mere event of non-specific DNA fragmentation is incompatible with our results and it seems more likely that association of the enzyme protein with specific base sequences can exert a regulatory effect on enzymatic activity.

The availability of chemically well defined octameric duplexes (A and C) as DNA substitutes provides a kinetic tool for the determination of the effects of enzyme inhibitors on the DNA binding site of the enzyme. The 6-amino derivative of 1,2-benzopyrone, the latter referred to as coumarin in [16] and shown to be an effective antitransformer drug, as demonstrated in fig.3 competitively inhibits at the octamer duplex A or C sites with an apparent K_i of 28 μM. Without the inhibitor at a fixed NAD^+ concentration, a Michaelis-Menten relationship exists between V_{init} and the concentration of the octadeoxyribonucleotide duplexes (lowest curve in fig.3) with an apparent binding constant of 1 μM. This value was obtained by varying the concentration of the octamer over a 10-fold range. When k_D values are calculated from plots where octamers are varied over a range of 10^4, lower k_D values are obtained (see table 1) reflecting the known sensitivity of double-reciprocal plots. The results shown in fig.3 identify a novel site of inhibitors, structurally unrelated to NAD^+ [16], which act at the DNA-binding site of poly(ADP-ribose) polymerase.

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