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Destabilization of Zn^{2+} coordination in ADP-ribose transferase (polymerizing) by 6-nitroso-1,2-benzopyrone coincidental with inactivation of the polymerase but not the DNA binding function*

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6-Nitroso-1,2-benzopyrone, an oxidation product of 6-amino-1,2-benzopyrone, binds to the DNA-recognizing domain of the ADP-ribose transferase protein and preferentially destabilizes Zn^{2+} from one of the two zinc finger polypeptide complexes present in the intact enzyme, as determined by the loss of 50% of $^{65}Zn^{2+}$ from the $^{65}Zn^{2+}$-isolated protein molecule, coincidental with the loss of 99% of enzymatic activity. The 50% zinc-deficient enzyme still binds to a DNA template, consisting of a 17-mer DNA primer annealed to M13 positive strand, resulting in the blocking of DNA synthesis by the Klenow fragment of Pol I. Auto-poly-ADP-ribosylated ADP-ribose transferase, which is the probable physiological state of this protein in intact cells, does not bind to primer-template DNA and does not block DNA synthesis by the Klenow fragment. On the basis of this in vitro model it is proposed that molecules which inhibit or inactivate ADP-ribose transferase in intact cells can induce significant alteration in DNA structure and replication.

Zinc finger; 6-Nitroso-1,2-benzopyrone; ADP-ribose transferase

1. INTRODUCTION

The major cell biological action of inhibitory ligands of the poly(ADP-ribose) polymerase activity of ADPRT (ADP-ribose) transferase; EC 2.4.4.30) consists of a cell cycle-dependent prevention of carcinogen-induced malignant transformation of human fibroblasts [1], conferring also carcinogen resistance [2], inhibition of malignant transformation in hamster embryo and mouse C3H10T1/2 cell cultures [3], deletion of transfected oncogenes from NIH 3T3 cells [4], suppression of the mitogenic stimulation of tumor promoters [5], inhibition of illegitimate DNA recombinations [6] and integration [7], induction of sister chromatid exchange [8] and the loss of certain amplified oncogenes [9,10]. The molecular structures of inhibitory ADPRT ligands vary from benzamide [1], substituted benzamides [3,5,7,9,10], 3-aminonaphthylhydrazide [6], isoquinoline, and quercetin, to coumarin (1,2-benzopyrone) [2]. We have demonstrated that 1,2-benzopyrone that binds to ADPRT at the DNA-recognizing domain inhibits both in cell cultures and in vivo the corticosteroid-activated malignant cell transformation of a rat cell line containing an oncogene construct [11]. The observed apparently pleiotropic action of inhibitory ADPRT ligands is not instantaneous and requires at least 1–3 cell cycles [1,2,11], indicating either a slow progression of cellular responses or possibly a biochemical transformation of certain ADPRT ligands to active molecular species, or both. It is known that 1,2-benzopyrone in vivo is readily hydroxylated in the 3 and 7 positions [12] and we found that the 7-hydroxy-1,2-benzopyrone does not inhibit ADPRT (unpublished results) which probably explains the transient nature of the antineoplastic effect of 1,2-benzopyrone in vivo [11]. However, substitution of an amino group in position 6 [13] produced 6-amino-1,2-benzopyrone (6-ABP) whose ADPRT inhibitory action and antineoplastic effect in cell cultures was not transient [14]. We have therefore further investigated the biochemical fate of 6-ABP and identified a novel oxidative metabolite, 6-nitroso-1,2-benzopyrone (6-NOBP), formed by mixed function oxidases of liver microsomes in vitro. The 6-NOBP molecule still inhibited ADPR-polymerase activity but also inactivated ADPRT by destabilization of one zinc ion from one of the two zinc fingers of this protein.

The following report deals with this mode of action

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Abbreviations: ADPRT, ADP-ribose transferase; 6-ABP, 6-amino-1,2-benzopyrone; 6-NOBP, 6-nitroso-1,2-benzopyrone; 6-NO_2BP, 6-nitro-1,2-benzopyrone; M13 ssDNA, bacteriophage M13mp18 single-stranded DNA; MES, 2-(*N*-morpholino)ethanesulfonic acid; GSH, reduced glutathione.

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of 6-NOBP in vitro, while possibly in vivo consequences of this reaction are subject to further investigation.

2. EXPERIMENTAL

6-Nitro-1,2-benzopyrone (6-NO_2BP) was purchased from Pfaltz & Bauer (Waterbury, CT), 2-mercaptoethanol, NAD^+, NADPH, nucleotides, sodium tungstate, hydrogen peroxide, Tris and MES buffers from Sigma (St. Louis, MO), Centricon 30 from Amicon (Beverly, MA), and HPLC-grade solvents and all other chemicals (reagent grade) from Fisher (Santa Clara, CA). [^{32}P]NAD^+ (250 Ci/mmol) and [^{32}P]dCTP (650 Ci/mmol) were obtained from ICN radiochemicals (Irvine, CA) and $^{65}ZnCl_2$ (8.24 mCi/mg) from Dupont/NEN (Wilmington, DE). M13mp18 single-stranded circular DNA, M13 sequencing 17-mer primer GTTTTCCCAGTCACGAC and the Klenow fragment of DNA polymerase I were from New England Biolabs (Beverly, MA).

6-ABP was prepared from 6-NO_2BP [13] and labeled in the 5-position with ^{3}H using methodology previously reported [11]. The specific activity of tritiated 6-ABP was 19 Ci/mmol. 6-NOBP was synthesized by the oxidation of 6-ABP (4 g/40 ml H_2O) at 22°C by 30% H_2O_2 (5 ml) with sodium tungstate (5.93 g, in 20 ml H_2O) for 1.5 h followed by extraction of the green product into ethyl acetate, washing with 0.1 N HCl, and evaporation of solvent. Recrystallization from warm ethanol gave 1.48 g (42%) of product with a λ_{max} at 750 nm, characteristic of monomeric arylnitroso compounds [15]. Mass spectrum = *m/z* (relative intensity): 175 (M^+, 100), 161 (16.88), 145 (33.77), 133 (10.38), 117 (56.09), 89 (79.71), 63 (57.13). High resolution data for the M^+ peak (calculated for $C_9H_5NO_3$): 175.0268; found: 175.0271 (deviation = 1.1 ppm). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm) from TMS: doublet (6.572 and 6.604) H-4 split by H-3; doublet (7.472 and 7.501) H-8 split by H-7; doublet of doublets (7.860/7.866 and 7.889/7.896) H-7 split by H-8 and finely split by H-5; doublet (7.910 and 7.942) H-3 split by H-4; doublet (8.308 and 8.315) H-5 finely split by H-7. UV/vis spectrum in ethanol, λ_{max} (ε): 750 nm (46.0), 316 nm (8.96 × 10^3), 274 nm (2.24 × 10^4). M.p.: compound polymerizes above 160°C, blackens and melts in the range of 325–340°C.

Electrophoretically homogeneous ADPRT was isolated from calf thymus by published methods [16,17], and for loading with $^{65}Zn^{2+}$ the enzyme (770 μg) was incubated in 500 μl of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM 2-mercaptoethanol with 45 μCi $^{65}Zn^{2+}$ for 120 h at 6°C, at which time the $^{65}Zn^{2+}$ content of the protein was in equilibrium with the $^{65}Zn^{2+}$ in the exchange buffer (see Fig. 3). The time course of Zn^{2+} loading of ADPRT was determined by simultaneously monitoring the specific radioactivity of ADPRT-bound $^{65}Zn^{2+}$ chemically [18] and radiochemically. Then ADPRT solution was concentrated to a protein concentration of 3 mg/ml in Centricon 30 and stored at −20°C in the presence of 20% glycerol. Ejection of $^{65}Zn^{2+}$ from $^{65}Zn^{2+}$-loaded ADPRT by 6-NOBP was assayed in 50 mM MES buffer (pH 6.0) containing 150 mM NaCl, 5 mM 2-mercaptoethanol and varying concentrations of 6-NOBP. Enzyme-bound $^{65}Zn^{2+}$ was measured by a filter binding assay [19]. Enzymatic assays for ADPRT activity [13,16] and for DNA synthesis on a primer annealed to M13 positive strand DNA were carried out by published methods [20].

The biological oxidation of 6-ABP was measured by incubating for 30 min (37°C) 10 ml of a reaction mixture composed of 0.1 mM [^{3}H]6-ABP (26.7 mCi/mmol), 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.25 mM KCl, 1 mM NADPH and 1 mg/ml of rat liver microsomes [21] which were isolated from Fisher male rats pretreated with Aroclor 1254 as reported [22]. The reaction was terminated by addition of an equal volume of cold acetone, and the mixture extracted with four 10-ml volumes of ethyl acetate, which combined were dehydrated over anhydrous sodium sulfate and evaporated to dryness by a stream of nitrogen. An aliquot of the residue dissolved in acetonitrile/water (1:1 v/v) was analyzed by HPLC with an elution system employing three solvents (A = 0.05 M potassium phosphate, pH 6.0; B = same as A + 30% methanol; C = 50% acetonitrile, 50% water) at a flow rate of 1.2 ml/min using a reversed-phase ODS column by a published method [23]. Analytical standards of 6-ABP, 6-NO_2BP and 6-NOBP eluted at 22.2 min, 33.5 min and 39.2 min, respectively. The identity of metabolites was established by their UV spectra and retention times which were identical with the authentic standards.

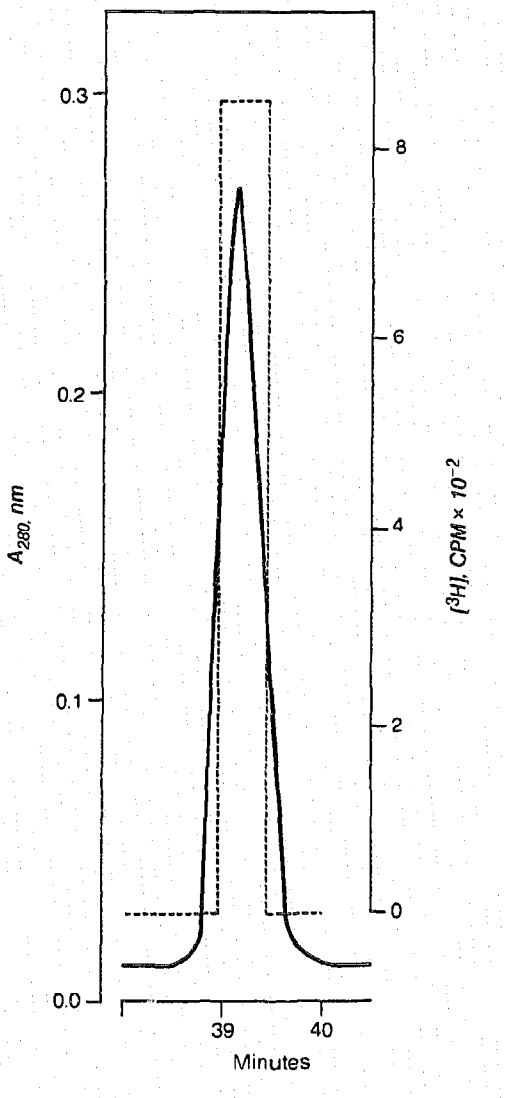

Fig. 1. Identification by HPLC of [^{3}H]6-NOBP as the main oxidation product of [^{3}H]6-ABP catalyzed by rat liver microsomes. The analytical standard 6-NOBP (solid line) was monitored by UV absorbance (left ordinate) and the enzymatic product (dashed line) by ^{3}H-radioactivity (right ordinate) with the chromatographic retention time shown on the abscissa. The UV spectra of the eluate peak and 6-NOBP standard, as measured by the diode-array detector, were identical (not shown). Details of the incubation system and HPLC procedure are given in section 2.

3. RESULTS

When [^{3}H]6-ABP was oxidized by microsomes for 30 min, HPLC analysis identified the main oxidation product (7% of 6-ABP) as 6-NOBP (Fig. 1), with 2% of 6-ABP oxidized to 6-NO_2BP formed simultaneously (not shown). Among the oxidation products of 6-ABP only 6-NOBP was an ADPRT inhibitor, and in an

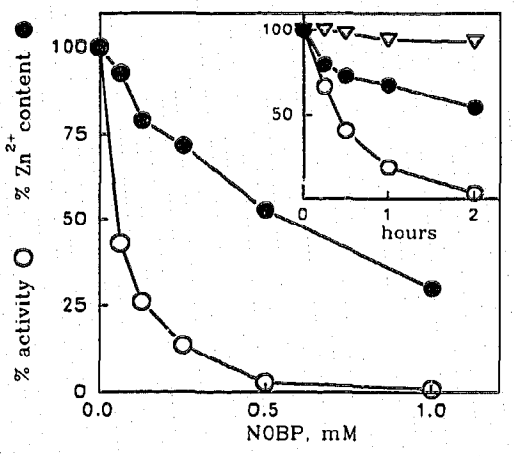

Fig. 2. The effects of incubation of ADPRT with 6-NOBP on the enzymatic activity and $^{65}Zn^{2+}$ content of the ADPRT protein. The ordinate shows the percentage changes in enzymatic activity (100% is 231 pmol ADPR/μg protein/min) and in $^{65}Zn^{2+}$ content (see Fig. 3) following 2 h incubation of $^{65}Zn^{2+}$-loaded ADPRT [17] with varying concentration of 6-ABP (abscissa) in a system composed of 50 mM MES-buffer (pH 6.0), 100 mM NaCl, 0.5 mM EDTA and 5.0 mM 2-mercaptoethanol and varying concentrations of 6-NOBP in a final volume of 10 μl at 25°C. A 1 μl aliquot was used for ADPRT assay (see section 2) and the remaining material for the radiochemical test for $^{65}Zn^{2+}$ content of ADPRT [19]. Closed circles indicate $^{65}Zn^{2+}$ content, open circles ADPRT activity. (Inset) The time course of the effect of 0.5 mM 6-NOBP on ADPRT activity (open circles) and $^{65}Zn^{2+}$ content (closed circles), while the open triangles indicate the protective effect of 0.5 mM $ZnCl_2$ on ADPRT activity in the presence of 0.5 mM 6-ABP. The pH 6.0 was chosen for 6-NOBP induced $^{65}Zn^{2+}$ ejection because its rate was maximal at that value but the induced $^{65}Zn^{2+}$ efflux from ADPRT occurs also at pH 7.0, with a rate reduced to 30%. All assays were done in triplicates, which agreed within 10%.

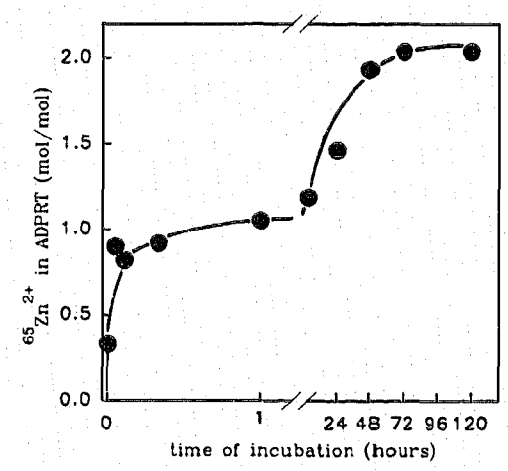

Fig. 3. Exchange of externally added $^{65}Zn^{2+}$ ADPRT-bound Zn^{2+} as a function of time of incubation. ADPRT (700 μg) was incubated at 6°C in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol to which 45 μCi of $^{65}Zn^{2+}$ (1.12 mCi/μmol) was added. The $^{65}Zn^{2+}$ and Zn^{2+} content of ADPRT were determined using published radiochemical [19] and chemical [18] methods. Assays were done in duplicates with ±10% variation.

ADPRT assay [13] with nM NAD as substrate and a synthetic octamer as coenzyme, 6-NOBP inhibited ADPRT with a K_i of 40 μM, as compared with K_i for 6-ABP of 28 μM, indicating that binding to ADPRT still occurs. In addition there was also an unexpected time-dependent inactivation of the poly(ADP-ribose) synthesizing activity of ADPRT by 6-NOBP. Incubation of ADPRT with increasing concentrations of 6-NOBP (Fig. 2) for 2 h in the presence of 5 mM 2-mercaptoethanol at 22°C and pH 6.0 resulted in a precipitous decrease of enzymatic activity coinciding with an ejection of $^{65}Zn^{2+}$ from the $^{65}Zn^{2+}$-loaded enzyme. The presence of 2-mercaptoethanol protected −SH groups of ADPRT against oxidation by the nitroso compound and favored a selective attack on the zinc finger complexes of ADPRT. Coincidental with an almost total loss of polymerase activity nearly half of the Zn^{2+} content of ADPRT was ejected by the incubation with 6-NOBP, an effect which was not altered by added coenzymic DNA (cf. [16]). Either 0.5 mM $ZnCl_2$ or $CdCl_2$ protected against inactivation by 6-NOBP (top curve, inset Fig. 2), but no protection was exerted by Fe^{2+}, Co^{2+}, or Mg^{2+} (not shown). The 6-NOBP-induced zinc loss was reversible in vitro, but reactivation, within the same time frame as Zn^{2+} ejection, required simultaneous incubation with added Zn^{2+}, dithiothreitol and DNA (results not shown). A correct interpretation of the 6-NOBP-induced zinc ejection from preloaded ADPRT critically depends on equilibration between externally added $^{65}Zn^{2+}$ and both enzyme-bound Zn^{2+}. As shown in Fig. 3, this was indeed attained since both Zn^{2+} of the enzyme protein exchanged with $^{65}Zn^{2+}$, albeit with two apparently distinct rates, as determined by assaying the specific radioactivity of ADPRT-bound Zn^{2+} as a function of time of incubation with $^{65}Zn^{2+}$.

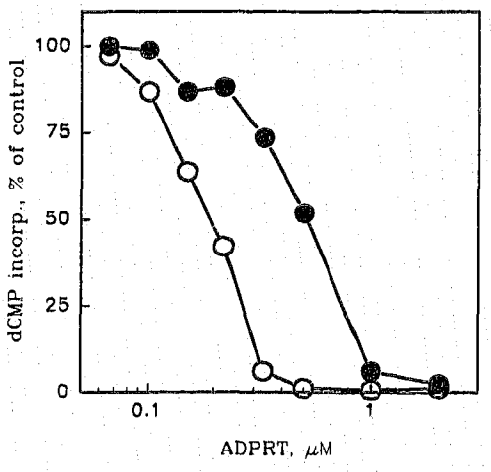

Fig. 4. Inhibition of DNA synthesis by varying concentrations of native ADPRT (closed circles) and 6-NOBP-treated Zn^{2+}-deficient ADPRT protein (open circles). The ordinate shows DNA polymerase activity as % of controls, assayed as [^{32}P]dCMP incorporation (30 min) by the Klenow fragment of DNA polymerase I with M13 ssDNA annealed template primer as reported [20]. The 100% value for Klenow polymerization is 112 pmol of nucleotides incorporated per 30 min in 50 μl reaction mix (25°C). Duplicates agreed within 10%.

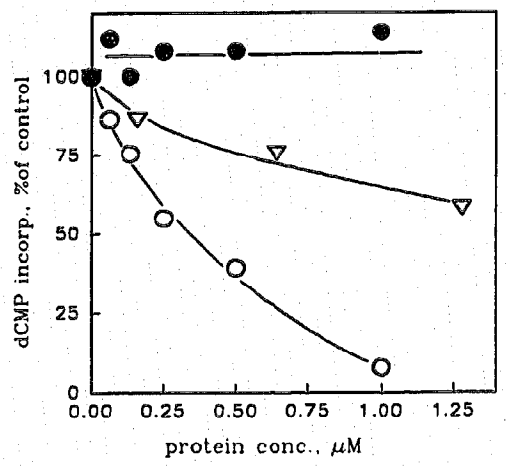

Fig. 5. The effects of ADPRT (open circles), auto-poly-ADP-ribosylated ADPRT (closed circles), and the isolated 29 kDa polypeptide (open triangles) on DNA synthesis on the M13 ssDNA annealed primer [20] by the Klenow enzyme. ADPRT was auto-poly-ADP-ribosylated by preincubation with 0.1 mM NAD^+ at pH 8.0 for 10 min at 25°C in the presence of all components, except the Klenow enzyme which was added after 10 min to initiate DNA synthesis, which lasted for 30 min. Triplicate assays agreed within 10%.

A quantitative measure of the binding of ADPRT to DNA termini was obtained by determining the inhibition of DNA synthesis on M13 ssDNA annealed primer [20] as illustrated in Fig. 4. Untreated ADPRT inhibited DNA synthesis as a function of ADPRT concentration and at 0.5 μM ADPRT nearly complete inhibition occurred (I_{50} = 0.17 μM). Removal of one Zn^{2+} ion per molecule of ADPRT reduced the inhibitory effect of ADPRT (I_{50} = 0.5 μM) but did not abolish it. ADPRT has no direct effect on the Klenow enzyme and in this system inhibition is solely due to the binding of ADPRT to 3' ends of DNA primers as also tested with coenzymic DNA (not shown). It is of interest that ADPRT concentration in nuclei is 1 μM [28] therefore these in vitro results have biological relevance. Based on the similarity to results with site-directed mutation of zinc finger FI [29] 6-NOBP appears to destabilize Zn^{2+} preferentially from FI. A direct testing of this conclusion will require a significant quantity of ADPRT mutated in FI, which has thus far been assayed in cell extracts only [29].

Since ADPRT in differentiated cells exists mainly in the auto-poly-ADP-ribosylated form [30], the effect of the in vitro auto-poly-ADP-ribosylated enzyme protein on DNA synthesis at the primer template site was also determined. As shown in Fig. 5 (upper curve) the automodified enzyme completely lost its inhibitory effect at the primer-template site. In separate experiments it was found that as few as 35 ADP-ribose units per molecule of ADPRT were sufficient to abrogate the inhibitory action of ADPRT on DNA synthesis (not shown).

The 29 kDa terminal polypeptide of ADPRT [31] which contains both zinc fingers [32,33] was slightly less than 50% as effective an inhibitor, when present in equimolar concentration with ADPRT (Fig. 5, middle curve), whereas the 36 kDa and 56 kDa polypeptide components of ADPRT [31] had no detectable inhibitory action at equimolar concentrations with the intact enzyme molecule (results not shown).

4. DISCUSSION

The oxidation of 6-ABP to 6-NOBP by microsomal mixed function oxidases (Fig. 1) may not be confined to one system since other O_2^- generating enzymatic reactions (e.g. in granulocytes) predictably can also perform this oxidation, a problem which is currently studied. In intact cells the steady-state concentration of enzymatically generated 6-NOBP should be very low because of its facile reduction to 6-ABP by GSH and other reducing systems, except at membrane-associated sites of its formation, presumably at the nuclear membrane where ADPRT also resides [24,25], a locus which is contiguous with the endoplasmic reticulum [26,27] containing oxidases. It is also possible that the cellular pharmacologic action of 6-ABP is probably a composite effect of ADPRT inhibition by 6-ABP itself to which a progressive inactivation of ADPRT by locally generated 6-NOBP is superimposed. The exact nature of this combined effect in cellular systems is not known. On the other hand, on the basis of present results the consequences of ADPRT inhibition and inactivation are predictable. Since the turnover of poly(ADP-ribose) in intact tissues is relatively slow ($t_{1/2}$ is between 2.6 and 4 h in vivo; cf. [39]), a progressive removal of the DNA-repelling [40] poly(ADP-ribose) from ADPRT by catabolic enzymes will take place following the inhibition and inactivation of poly(ADP-ribose) synthesis by ADPRT ligands. Thus a time-dependent emergence of de-ADP-ribosylated ADPRT occurs, which is then available for binding to specific DNA structures and complex cellular responses may ensue [1–11].

The most probable mechanism of the destabilization of Zn^{2+} from ADPRT by 6-NOBP is the oxidation of cysteine ligands [29] in the zinc finger peptide, forming disulfide and 6-hydroxylamino-1,2-benzopyrone, similar to the reactivity of other nitroso compounds [38]. As expected, other arylnitroso compounds can also destabilize Zn^{2+} in ADPRT, but among the molecules so far tested (nitrosobenzene, 4-nitrosophenol, 1-nitroso-2-naphthol-3,6-disulfonic acid), 6-NOBP proved to be the most effective, probably because of its binding affinity to ADPRT.

The facile exchange of $^{65}Zn^{2+}$ with the two enzyme-bound Zn^{2+} ions of ADPRT, as illustrated in Fig. 3, is not a universal property of all zinc finger proteins, which frequently require pretreatment with organomercurials [34,35] for Zn^{2+} release. However in both cases cited [34,35] only one of the zinc fingers was destabilized by *p*-hydroxymercuriphenylsulfonate, similar to our observations with 6-NOBP in ADPRT. In our experience

p-hydroxymercuribenzoate removed both Zn^{2+} ions of ADPRT and denatured ADPRT, in contrast to 6-NOBP which reversibly destabilized one Zn^{2+} ion (Fig. 2). The apparently biphasic Zn^{2+} exchange in ADPRT (Fig. 3) may suggest dissimilarity between the two zinc fingers. Dissimilarity of zinc fingers within the mammalian glucocorticoid receptor was earlier indicated by their differing NMR relaxation times [36]. The need for individual assessment of zinc binding polypeptide structures has been recently emphasized [37]. If the in vitro detectable relative ease of Zn^{2+} exchange exists in cellular systems, ADPRT may be a biological Zn^{2+} sensor, a question which requires further studies.

The 29 kDa polypeptide of ADPRT by itself has similar but weaker template inhibitory action than ADPRT (Fig. 5), suggesting that the second DNA binding polypeptide of ADPRT (36 kDa; cf. [31]), which by itself is not inhibitory, in the intact ADPRT molecule apparently reinforces the binding of the adjacent zinc finger domain to DNA termini. The significance of the second (36 kDa) DNA binding domain of ADPRT [31] has been emphasized by the recognition of a helix-turn-helix motif in the cDNA of ADPRT, coding for the 36 kDa polypeptide [41].

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