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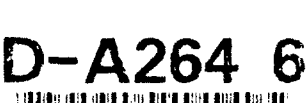

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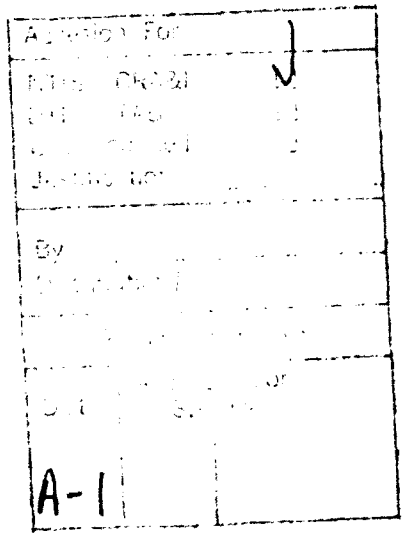

BY:

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Project Director

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INHIBITION OF DNA BINDING BY THE PHOSPHORYLATION OF POLY ADP-RIBOSE POLYMERASE PROTEIN CATALYZED BY PROTEIN KINASE C

Abstract:

Purified type II (β) and type III (α) protein kinase C phosphorylates highly purified polyADP-ribose polymerase *in vitro* whereby 2 mols of phosphate are transferred from ATP to serine and threonine

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Abbreviations

TCA, trichloroacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; TBE, Tris-boric acid-EDTA buffer; BSA, bovine serum albumin

residues present in the 36 and 56 kDa polypeptide domains of the polymerase protein. Calf thymus DNA was a non-competitive inhibitor of the protein kinase C catalyzed phosphorylation of polyADP-ribose polymerase. Coincidental with the phosphorylation of the protein the polymerase activity and DNA binding capacity of polyADP-ribose polymerase were inhibited. These *in vitro* findings may have possible cell biological significance in cellular signal transduction.

Introduction:

Poly ADP-ribose polymerase (ADPRT) is a highly abundant nonhistone nuclear protein of higher eukaryotes (1,2) which exhibits a cellular differentiation-dependent variation in catalytic activities, consisting of NAD-glycohydrolase and ADP-ribose polymerase (3). Besides enzymatic activities, ADPRT possesses significant colligative properties towards DNA termini and certain internal DNA structures (4,5,6,7) as well as towards a number of DNA binding nuclear proteins which can serve as polyADP-ribose acceptors (8-13). The catalytic activity of ADPRT is enhanced more than 100-fold by DNA possessing free termini and this activation is

relatively nonspecific, i.e. various fragmented DNAs can serve as "coenzymes". However even with synthetic octadeoxyribonucleotides there is a secondary influence of base sequences on catalytic activation (14). In contrast to DNA termini, circular DNAs also bind ADPRT, an association that results in topological changes in circular DNA (7). Whereas the binding site of DNA termini has been localized to the 29 kDa basic terminal polypeptide at zinc fingers (15), the mode of association of ADPRT with rare internal regions is as yet not fully understood. Several attempts have been made to correlate the function of ADPRT with cellular signal transduction, a possibility which is attractive since ADPRT may serve as a nuclear DNA binding element to receive metabolic signals coming from extranuclear cell compartments. Indirect evidence for a regulatory function of ADPRT (16,17) was obtained by an increase of ADPRT activity following the addition of phorbol esters to human fibroblasts. It was also shown that protein kinase C (PKC) can phosphorylate ADPRT in vitro (18).

Participation of PKC as a signal transducer from the cell membrane to a DNA binding nuclear enzyme (e.g. ADPRT) is made feasible by a drug-induced translocation process of

PKC to the nucleus (19,20,21), and it is of particular interest that topoisomerase II which is also an acceptor of polyADP-ribose (13) can be phosphorylated by PKC (22,23) resulting in activation of topoisomerase II whereas poly ADP-ribosylation produces enzyme inhibition (13). We report here that phosphorylation of ADPRT at specific sites interferes with the DNA binding of this protein.

Materials and Methods:

ADPRT of greater than 95% purity was isolated from calf thymus by established techniques (24) and ADPRT activity was measured as reported earlier (14). The purification of type II (β) and type III (α) isoenzymes of PKC from rabbit thymus cells was carried out as described previously (25). The assay of PKC activity was performed in a standard reaction mixture (200 µl volume) consisting of 50 mM TRIS-HCl (pH 7.4), 10 mM $MgCl_2$, 20 mM (γ-^{32}P)ATP, (0.5-2x10^6 cpm/assay). PKC was activated by adding Ca^{2+} (0.1 mM final), phosphatidylserine (25 µg/ml) and diacylglycerol (250 ng/ml) to the reaction mixture. The specific oligopeptide substrate AAASFKAKK-amide (0.7 mg/ml) was used as phosphate acceptor as described earlier (26). One unit of PKC

activity is defined as the amount of enzyme catalyzing the incorporation of one nmol of phosphate per min into the oligopeptide.

Phosphorylation of ADPRT was carried in the standard reaction mixture at 37 °C for 5, 30 and 60 min as indicated in Results. The reactions were stopped either by adding 1.5 ml of 15% TCA to the reaction mixture or by chelating Ca^{2+} with 2 mM EGTA, as in the case of PAGE retardation assays and when degradation of ADPRT by plasmin was performed.

To characterize the phosphate acceptor amino acid, ADPRT (4 μg) was phosphorylated in the standard reaction mixture for 30 min with 0.4 units of PKC and the reaction was stopped by TCA. Bovine serum albumin (10 μg) was added as carrier and the precipitated proteins collected by centrifugation (6000 x g, 10 min at 4°C). The ether-washed proteins were hydrolyzed in 6N HCl for 3 h at 110°C and HCl was removed by repeated freeze-drying and authentic Ser-P and Thr-P were added as standards followed by paper electrophoresis (27). The paper strips were ninhydrin-stained, dried and autoradiographed. The amount of phosphorylated amino acids were determined by liquid scintillation

spectrometry.

The DNA binding ability of ADPRT was assayed by a PAGE retardation method as described earlier (6). The 342 bp PstI-PstI fragment from the first coding exon of the Ha-ras gene was used throughout these experiments as DNA. This DNA was subcloned into PBR322 vector, isolated, dephosphorylated and end-labeled with T4-kinase by standard methods (28).

Localization of the polypeptide domains of ADPRT containing phosphorylated amino acids was carried out as follows. Ten μg of ^{32}P-labeled ADPRT was digested with 0.1 μg of plasmin for varying time periods (see legend to Fig 1) and the polypeptides obtained were separated by SDS-PAGE as described (29).

Results and Discussion:

In agreement with previous results (18) ADPRT was phosphorylated in vitro by activated type II (β) or type III (α) isoforms of PKC (see lane 0 of Fig 1). The ADPRT molecule was heavily labeled by the activated PKC isoenzymes in

contrast to the control system that contained no phosphatidylserine and diacylglycerol (not shown). The phosphorylation of ADPRT coincided with a loss of 80-85% of the polymerizing activity of ADPRT (data not shown).

The stoichiometry of phosphorylation of ADPRT by 0.4 U of PKC during a reaction time of 60 min was between 1.6-1.9 mol phosphate per mol ADPRT protein. Localization of the phosphorylation within the ADPRT molecule was accomplished by the isolation of proteolytic fragments following the digestion of ADPRT with plasmin (29). Results are shown in Fig. 1. The 29 kDa fragment is derived from the N-terminal part of ADPRT and contains the two zinc finger structures (15), whereas the 36 kDa domain contains large amounts of basic amino acids and participates in the internal DNA binding of ADPRT (4). The 56 kDa C-terminal fragment of ADPRT bears the catalytic site and most of the ADP-ribose acceptor sites (30).

As seen from Fig. 1, ^{32}P-labeling was always found in the 36 kDa and 56 kDa fragments of the enzyme. Phosphorylation of the two distinct fragments and the stoichiometry of phosphate incorporation are consistent with two sites of phosphorylation.

The apparent distribution of ^{32}P between threonine and serine residues of ADPRT were not equal as shown by direct analysis (see Methods). Acid hydrolysis was limited to 3 h which degraded 10-20% of ADPRT. More extensive hydrolysis degrades the phosphate bonds. Under our conditions ^{32}P content in cpm was 1560 in Thr-P and 6180 in Ser-P. Somewhat similar results were obtained when lipocortin I, another P acceptor from ATP, catalyzed by PKC (2968 in Thr-P, and 4370 per Ser-P) was used. These results tend to suggest that serine is a better P acceptor, but it cannot be ruled out that the reason for this inequality may be due to structural factors which make Ser more accessible to ATP.

The lysine-rich DNA binding 36 kDa fragment contains one of the two sites of phosphorylation by PKC in ADPRT. As we have shown that blocking of ADPRT by methyl acetimidate at Lys residues abolishes the activation of ADPRT by DNA (31). We assumed that phosphorylation in this polypeptide domain may also alter the binding of DNA of ADPRT. As illustrated in Fig. 2 the phosphorylation of ADPRT resulted in the loss of DNA binding capacity of the enzyme as assayed by gel retardation. PKC itself in the absence of its activators did not interfere with the DNA binding of ADPRT.

As was expected, the binding of DNA to ADPRT inhibits the phosphorylation of the enzyme by PKC. The Km values for PKC and its isoforms are slightly dissimilar: $3\pm 1x10^{-7}$ M for the type III isoenzyme and $1.5\pm 0.8x10^{-7}$ M for the type II isoenzyme. Both are about an order of magnitude lower than the previously published values for mixture of PKC isoenzymes (18) clearly indicating that ADPRT is a good substrate of PKC. When the incubation was carried out in the presence of DNA (at a saturating concentration for ADPRT) only a very low level of phosphorylation was detected. DNA behaved as a non-competitive inhibitor of phosphorylation by PKC with respect to ADPRT, but DNA itself had no effect on PKC as also tested with the specific oligopeptide substrate of PKC (26) and therefore the inhibition by DNA is due to a ADPRT-DNA interaction (Fig 3). Phosphorylation of both the 36 and the 56 kDa proteolytic fragments were equally inhibited (results not shown), which can be explained by wrapping of DNA around the ADPRT molecule (32). The findings shown in this paper are evidence that the phosphorylation of ADPRT by PKC inhibits its DNA binding ability and this mechanism could connect major routes of signal transduction (33,34,35) to the gene regulatory function

of ADPRT (30).

The translocation of PKC into the nucleus (19-23) represents a feasible mechanism of molecular interaction between PKC and ADPRT but the cellular significance of this hypothetical signal transfer step in the nucleus remains to be clarified.

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Figure 1. Autoradiogram of polypeptides obtained by plasmin digestion of phosphorylated ADPRT.

ADPRT (4 μg) was ^{32}-phosphorylated by 0.4U of type III (α) PKC for 30 min and samples digested with plasmin for the time periods shown in the abscissa, as described in Materials and Methods.

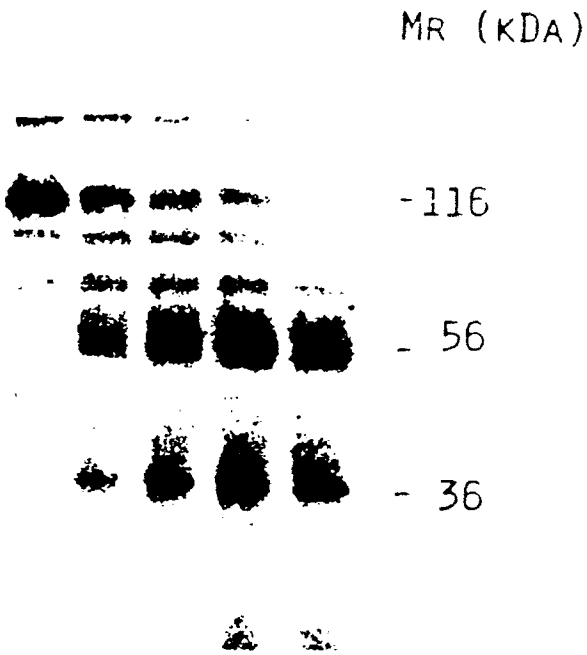

MR (KDA)
-116
- 56
- 36
0 2 5 15 30
TIME OF PLASMIN
DIGESTION (MIN)

Figure 2. The effect of phosphorylation of ADPRT on the DNA binding capacity of the protein.

Aliquots of ADPRT were incubated with PKC 0.4 U of type III (α) either in the presence (O-O) or in the absence (∇-∇) of 25 μg/ml of phosphatidylserine, 250 ng/ml of DAG and 0.1 mM of Ca^{2+} for 30 min at 37°C in a standard reaction mixture containing 20 mM ATP. As a control ADPRT was incubated in a reaction mixture where PKC was omitted (●-●). The reaction was quenched by EGTA. ^{32}P-labeled DNA ($2x10^4$ cpm in 5 μl; 10^7 cpm/μg DNA) was added to each sample dilution and incubated for 10 min at 23°C. The incubation was terminated by addition of 3 μl of loading buffer, and aliquots (20-μl) were loaded onto a 5% polyacrylamide gel and electrophoresed for 3 h at 60V in TBE buffer. The gel was dried and autoradiographed. The amount of protein bound DNA was determined by liquid scintillation spectrometry.

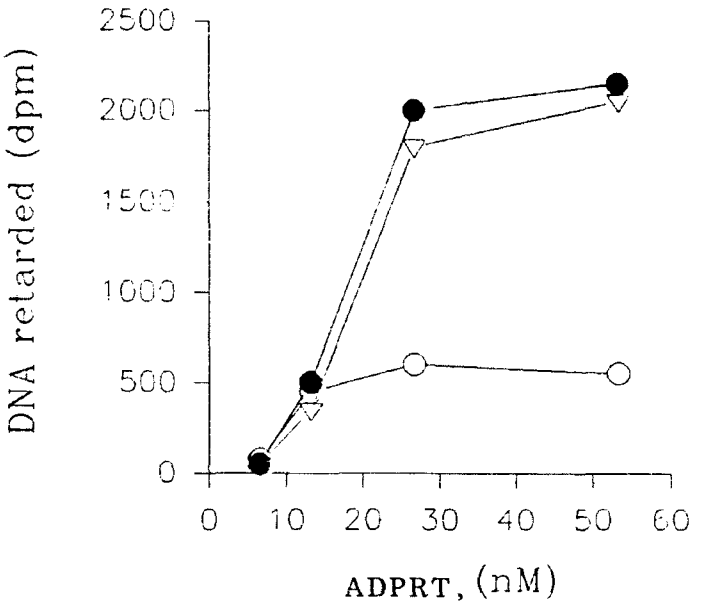
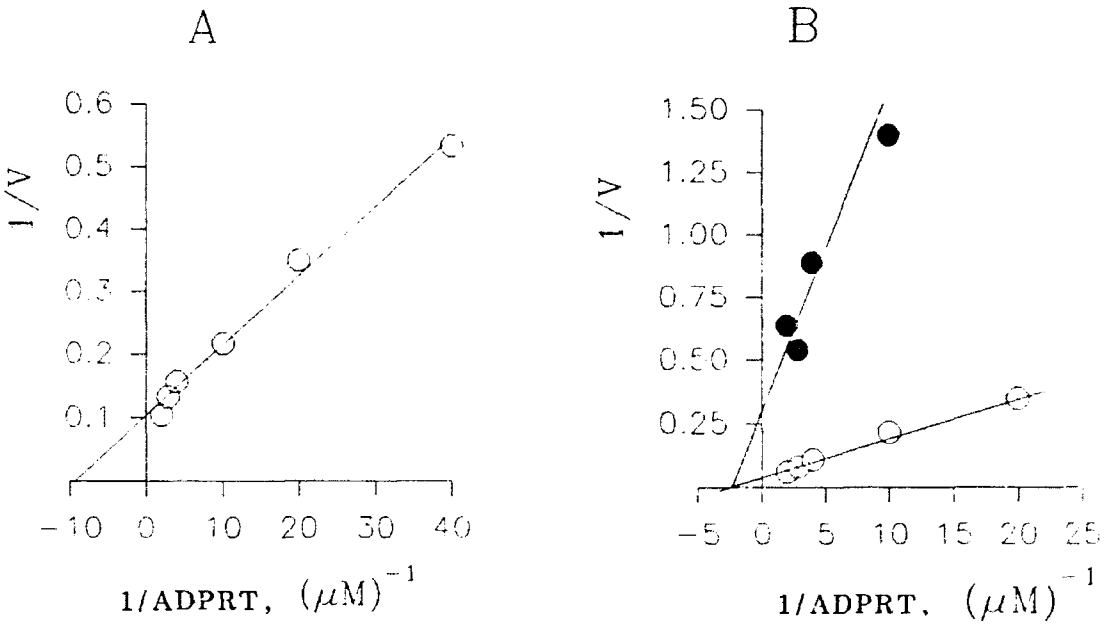

DNA retarded (dpm)
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
ADPRT, (nM)

Figure 3. Kinetics of ADPRT phosphorylation in the absence and in the presence of DNA.

Phosphorylation of ADPRT was performed either with 0.22U of type II (panel A) or 0.4U of type III (panel B) isoforms of PKC (O-O). In case of type III enzyme the phosphorylation was carried out also in the presence of 125 μg/ml calf thymus DNA (●-●). After incubation at 37°C for 5 min the reaction was stopped by addition of 1.5 ml of 15% TCA and 10 μg of carrier BSA was added and incubated 30 min on ice. The samples were centrifuged and the precipitated proteins dissolved into 40 μl of sample buffer. Aliquots (15 μl) were loaded onto 10% SDS-PAGE gel in duplicates. After electrophoresis, the ^{32}P-ADPRT bands were analyzed for ^{32}P and results expressed as pmol ^{32}P incorporated per min.


A
B
1/V
0.6
0.5
0.4
0.3
0.2
0.1
−10 0 10 20 30 40
1/ADPRT, $(\mu M)^{-1}$
1/V
1.50
1.25
1.00
0.75
0.50
0.25
−5 0 5 10 15 20 25
1/ADPRT, $(\mu M)^{-1}$

SPECIFIC BINDING SITES FOR HISTONES ON THE POLY(ADP-RIBOSE) POLYMERASE PROTEIN

Abstract:

Mono and dimeric forms of poly(ADP-ribose) polymerase enzyme (ADPRT) protein and a complex between the enzyme protein and histone H2B were identified by chemical cross linking and gel electrophoresis. Only the basic, DNA-binding

---

Abbreviations

ADPRT : poly(ADP-ribose) polymerase; coDNA : coenzymic DNA; PVDF : polyvinylidene difluoride; DTT : dithiotreitol; TCA : trichloroacetic acid; PBS : phosphate buffered saline; TE : Tris-EDTA; PMSF : phenylmethyl-sulfonyl fluoride; BSA : bovine serum albumin; Tween 20 : polyoxyethylene-sorbitan monolaurate; MNNG : N-methyl-N'-nitro-N-nitrosoguainidine; HPLC : high pressure liquid chromatography; PTH- : phenylthiohydantoin-; CNBr : cyanogen bromide; CAPS : 3-cyclohexylamino-1-propanesulfonic acid.

polypeptide component of ADPRT binds histones, as assayed in electroblots. Polypeptides of ADPRT, obtained by CNBr fragmentation, were assayed for their histone-binding capacity with the aid of histone-Sepharose affinity column. A combination of elution with a salt gradient, and identification by N-terminal sequencing defined two histone binding polypeptide domains, one between amino acid residues 186 and 290, which is close to zinc finger II, and a second 446-525, which is part of the automodifiable region and vicinal to the catalytic domain of the enzyme protein.

Introduction:

Poly(ADP-ribose) Polymerase (ADPRT, E.C.2.4.2.30) is an abundant nuclear protein of higher eukaryotes (1, 2) that exhibits at least two distinct catalytic functions: ADP-ribose polymerizing and NAD glycohydrolase activities which in turn are also regulated by the state of differentiation of particular cell types (3). The biochemical role of ADPRT in living cells in most probably related to both catalytic and colligative properties of this protein but these activities are difficult to assess in intact cells. Immunochemical estimation

of native and automodified ADPRT in intact cells in culture (AA-2 and MT-2 cells) indicated that only 4% (for AA-2) or 20% (for MT-2) of ADPRT was auto-ADP-ribosylated, thus a significant portion of this protein is available for macromolecular associations (4). Indeed recent results (5) show that *in vitro* ADPRT binds to and significantly stimulates DNA polymerase a but not b. Although these were *in vitro* model experiments, their cellular significance cannot be ruled out. Microinjection of the 46 kDa DNA-binding polypeptide fragment of ADPRT into human fibroblasts inhibits MNNG-induced unscheduled DNA synthesis (6), further supporting the contention that protein-protein or protein-DNA interactions involving ADPRT may have biological consequences. Poly-ADP-ribosylation inhibits the Ca^{2+}/Mg^{2+}-dependent nuclear DNA endonuclease *in vitro* (7) and we have shown that drug-induced derepression of this endonuclease produces selective apoptosis in leukemia cells (8).

Histones are well known activators of ADPRT (9) and their poly-ADP-ribosylation *in vivo* (10) appears to suggest that catalytic rates *in vitro* may be increased by histones serving as efficient polymer acceptors (11, 12). Results in this paper

suggest that the histone-ADPRT interaction is more complex, inasmuch as two specific polypeptide domains exist in ADPRT that bind histones.

Materials and Methods:

More than 95% homogeneous ADPRT and coenzymic DNA were isolated from calf thymus as reported (13, 14). The synthetic octadeoxyribonucleotide duplex 5'GCATGCAT3' (octamer C) was synthesized as described (15). The commercial sources of reagents were as follows: CNBr-activated Sepharose 4B from Pharmacia (Piscataway, NJ), Affi-Gel 10 from Bio-Rad (Richmond, CA), [^{32}P]-NAD^{+} and the [^{125}I]-Bolton-Hunter reagent from ICN (Irvine, CA), and Centricon concentrators from Amicon (Danvers, MA). All other chemicals used were of the highest purity available. The association of the histone H2B with ADPRT was shown by crosslinking with dimethyl-3,3'-dithiobispropionimidate as reported (16).

Partial digestion of ADPRT with chymotrypsin (17) was done

as follows. ADPRT (2 mg/ml in 50 mM Tris-HCl, 200 mM NaCl, 10 mM 2-mercaptoethanol, pH 8.0) was digested with 3.3 mg/ml chymotrypsin for 30 min. at 25°C, then the reaction stopped with 1 mM PMSF and the polypeptides isolated by HPLC as reported (18). The basic N-terminal 64 kDa polypeptide eluted at 0.45 M NaCl from MonoS cation exchanger column. The C-terminal 56 kDa polypeptide, which did not bind to the cation exchanger, was isolated on a benzamide-Affigel 10 affinity column as described earlier (14).

For the identification of ADPRT on acid urea gels (19), the enzyme was labeled with ^{32}P by mono-ADP-ribosylation with 100 nM [^{32}P]-NAD^+ (20). The isolated mono-ADP-ribosylated ADPRT protein was then mixed with 20 fold excess unlabeled ADPRT and this, in terms of specifically labeled protein, diluted ADPRT was used for crosslinking and was applied to gels (Fig. 1). Control experiments indicated that the low percentage of ADP-ribosylation did not influence the electrophoretic mobility of ADPRT when compared to the unmodified enzyme protein (not illustrated).

The polypeptides to be labeled with ^{125}I were dissolved in

25-50 ml of 50 mM borate buffer (pH 8.5) and pipetted into Eppendorf centrifuge tubes, which contained 2 ml [^{125}I]-Bolton-Hunter reagent (1888 Ci/mmol, 33 mCi in benzene-dimethylformamide) previously evaporated to dryness by a stream of N_2. After mixing by aspiration into a micropipette several times, the iodination reaction was allowed to proceed at 6^{o}C overnight. The unreacted Bolton-Hunter reagent was quenched with 5 ml of 1 M Tris-HCl (pH 9.0) and the reaction mixture gel-filtered through an 1.5 ml Sephadex G25 (fine) column equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1 % gelatin. The exclusion volume contained the iodinated polypeptides. The incorporation of ^{125}I was in the range of 3 to 5 %.

ADPRT and its chymotryptic fragments were separated by 10% SDS-PAGE (21), and histones or CNBr-fragments of ADPRT on a 17.5% acrylaminde-SDS system as described (22).

Electroblotting was carried out in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) / NaOH (pH 11.0) buffer containing 15% methanol for 90 minutes.
Amino acid sequencing was performed on excised PVDF

membranes (ProBlott membrane, Applied Biosystems, Foster City, CA) containing the transblots of peptides with the Model 470-A gas-phase sequencer and an on-line 120A PHT-analyzer (Applied Biosystems, Foster City, CA) according to a published method (23).

The binding of polypeptides to nitrocellulose membranes was determined as follows. Membranes containing electroblotted peptides were soaked in "renaturation buffer" (50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT and 0.3% Tween 20, pH 8.0, c.f. 24) for 1 hour then blocked by incubation with 2% defatted powdered milk (dissolved in PBS containing 0.1 mM PMSF) for 6 hours at room temperature. The membranes were then washed with a "low salt buffer" (50 mM Tris-HCl, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.05% BSA and 0.05% Tween 20, pH 7.5). The ^{125}I-labeled peptides (4-8 mg, 0.5-1x10^6 cpm) were incubated in sealed plastic bags with the membranes (transblots) in 5 or 10 ml of "low salt buffer" (the volume depending on the size of the membrane) overnight at 6^{o}C with gentle agitation. The membranes were then washed four times with the "low salt buffer" at room temperature and once with TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) at room temperature. After drying, the

membranes were exposed to X-ray films and developed.

Cyanogen bromide fragments of ADPRT were prepared by the following technique. Purified ADPRT (200 mg) was precipitated with 20% TCA, and pelleted by centrifugation, washed with 70% ethanol and dried. The dried protein was dissolved in 100 ml of 88% formic acid, and 300 ml of 0.1 M HCl and 20 ml of CNBr (100 mg/ml in ethanol) were added and the mixture allowed to stand for 48 hours at room temperature. Then the solvent was evaporated by freeze-drying and the residue dissolved in 100 ml of "renaturation buffer".

Histones-Sepharose affinity matrix was prepared from CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) and histones (Type II-AS, Sigma, St Louis, MO) according to the manufacturer's prescription (3 mg histones per ml of packed gel). The amount of histones bound to the matrix was determined in 0.1 ml aliquots of the settled resin by a published method (25). On the average 0.9-1.2 mg of histones were covalently bound per 1 ml packed bed of gel matrix.

Results and Discussion:

The binding of histone H2B (mol. mass 13.8 kDa) to ADPRT was first determined by chemical crosslinking (16) as illustrated in Fig 1. In lane 1 the monomeric ADPRT (116 kDa) and its dimer (200 kDa) separated. When histone H2B was added, a new band appeared that was located between the mono and dimeric form of ADPRT (lane 2). Chemical reduction of the cross linker (16), as would be predicted, yielded the monomer of ADPRT only as the single [^{32}P]-labeled protein band. The unlabeled H2B, commensurate to its low mol. mass, migrated below the ADPRT bands and it is not visible in this gel, except by Coomassie staining (not shown). The H2B histone species could be replaced by any other histones indicating a non selective binding of histones to ADPRT. We demonstrate the behavior of H2B only because this species was available to us in a relatively homogenous form.

The binding of polypeptides derived from ADPRT by chymotryptic digestion to radioiodinated core histones was assayed on nitrocellulose membranes. The separation of chymotryptic polypeptides of ADPRT, mainly representing a

mol. mass of 64 kDa, 56 kDa and 42 kDa are shown (Coomassie stain) in lane 1 of Fig. 2, coinciding with published results (17). When the chymotryptic polypeptides were transblotted onto nitrocellulose membranes and incubated with radioiodinated core histones, only the basic polypeptide of ADPRT (64 kDa) indicated histone binding (Fig. 2, lane 2).

The correctness of this conclusion was tested also by "opposite" labeling, i.e. radioiodination of the major chymotryptic polypeptides of ADPRT (i.e. the 56 and 64 kDa fragments) and their binding to transblotted core histones. In each experimental model the same results as depicted in Fig. 2 were obtained, i.e. only the basic half of ADPRT bound to histones, regardless which species was labeled with ^{125}I. In Fig. 3, lanes 1 and 2 show Coomassie blue stained histones (mixed) at two concentrations (2 mg in lane 1, 8 mg in lane 2). Lanes 3 and 4 illustrate that the 56 kDa polypeptide of ADPRT (the catalytic domain) does not bind to 2 and 8 mg transblotted histones at all. The binding of 4 mg of labeled basic 64 kDa polypeptide to 2 and 8 mg transblotted histones can be seen in lanes 5 and 6 respectively, whereas in lanes 7 and 8 as a positive control, the binding of labeled intact

ADPRT protein (8 mg) is seen, the intact protein bound to transblotted histones in the same manner as to the 64 kDa basic polypeptide (compare lanes 7, 8 to 5, 6).

A more detailed localization of histone binding sites on ADPRT was achieved by percolating CNBr-generated peptide fragments of ADPRT through a histone-Sepharose affinity matrix and identifying the retarded fragments. From the known amino acid sequence of bovine ADPRT (26) it is predictable that CNBr fragments of ADPRT in the region between zinc finger II and the catalytic domain, yields peptides of 3-18 kDa size, large enough to assume a conformation sufficient to exhibit similarity in binding ability to the native protein. In contrast, CNBr fragmentation of the zinc finger region produces some very short peptides. However, it is already known that the zinc finger participates in binding of DNA termini (27). Adsorption to histone matrix, followed by elution with stepwise increasing ionic strength also permits the estimation of relative binding strength of polypeptides to the histone matrix. This experiment is illustrated in Fig. 4. Electrophoretic separation of CNBr fragments is shown in Fig. 4 lane 1. In lane 2 the nonadsorbed (follow-through) fragments, eluted in 50 mM

Tris.HCl (pH 8.0) containing 10 mM 2-mercaptoethanol, are seen. Lanes 3 to 7 show fragments emerging at 50 (lane 3), 100 (lane 4), 200 (lane 5), 400 (lane 6) and 1000 mM NaCl (lane 7) added to the elution buffer. The polypeptide with an apparent mass of 14 kDa eluted between 50 and 200 mM NaCl accompanied (lanes 3 and 4) by a broader band which corresponds to proteolytic breakdown of this fragment. This proteolytic degradation product frequently accompanies ADPRT during its isolation and contains amino acid sequences susceptible to proteases. The 14 kDa polypeptide was identified by sequencing and corresponds to residues 186-290 on the sequence of ADPRT (105 residues, MW = 11,790), its N-terminal being GFSVL... . This polypeptide is located between the 29 and 36 kDa domains of ADPRT obtained by digestion with plasmin (18). It is only 22 residues downstream of the second zinc finger as seen in the domain diagram (Fig 5). We also sequenced the "high affinity" fragments (11 kDa and 18 kDa, shown in lane 6). The N terminal of the 18 kDa peptide was XXLTLGXLSQ..., this sequence positions the peptide on the bovine enzyme to 396-525 (130 residues, MW = 14,950). Sequencing of the 11 kDa fragment gave an N-terminal sequence of XEVKEANIRV... . The position of this peptide is the 446-525 (80 residues, MW

= 8,790). These two polypeptides thus overlap, the larger includes the smaller one, adjacent to the 56 kDa catalytic domain (Fig. 5) and are part of the automodifiable region of the enzyme (28).

The quantity of the "high affinity" fragments was always less than that of the 14 kDa fragment. In fact, in some experiments they appeared to be absent. One probable explanation for this phenomenon is that these polypeptides might not be renatured as readily as the 14 kDa fragment under the conditions used here, and may not bind to the affinity matrix.

Our results identify two histone binding regions, one between residues 186 and 290, which is close to the second zinc finger, and a second histone binding sequence that is between residues 446 and 525 which is within the automodifiable portion of ADPRT. The biochemical significance of the two histone binding regions within the N-terminal half of ADPRT is the subject of further study. The experimental procedures described here are readily applicable for the identification of the binding sites of other ADPRT associating proteins.

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Fig. 1. Crosslinking of histone H2B to ADPRT with the reversible homobifunctional crosslinker dimethyl-3,3'-dithiobis-propionimidate. Two mg of ADPRT were autolabeled by incubating with 100 nM [^{32}P]-NAD^{+} and 40 nM octamer C for 20 min. at 23°C in a volume of 15 ml. The reaction was terminated with 100 mM benzamide and the mixture was rendered 1 M with respect to NaCl. ADPRT was separated from the small molecules by using a Centricon 30 device. Unlabeled ADPRT (20 pmoles) was mixed with 1 pmol of the [^{32}P]-labeled species above (5000 cpm) and with 2 mg of H2B in a total volume of 14 ml, containing 100 mM HEPES buffer (pH 8.0). To this mixture dimethyl-3,3'-dithiobispropionimidate crosslinker was added to a final concentration of 10 mM and incubated for 30 min. at 23°C. The reaction was quenched with 25 mM hydrazine sulfate, and an equal volume of acidic SDS-PAGE sample buffer was added. Gel electrophoresis was performed on a 10% acidic urea-SDS-PAGE gel. The gel was stained with Coomassie blue, dried and autoradiographed. Lane 1, crosslinked ADPRT only; Lane 2, crosslinked ADPRT + histone H2B; Lane 3 same as Lane 2, but sample boiled for 2 min. in the presence of 1% 2-mercaptoethanol.


Mr (kDa)
200
116
98
1
2
3

Fig 2. Binding of labeled histones to transblotted chymotryptic fragments of ADPRT. The digestion of ADPRT, separation of polypeptides, electroblotting onto nitrocellulose membrane and the procedure for binding histones are described in Materials and Methods. Lane 1: Coomassie stained peptides, lane 2: autoradiography of bound histones to transblotted peptides.


Mr(kDa)
1
2
205
116
98
67
45
29

Fig 3. Binding of ADPRT and its N-terminal or C-terminal moieties to transblotted histones. Procedures described in Materials and Methods. Lanes 1 and 2 Coomassie stained histones, all the other lanes autoradiographs. In lanes 3 and 4 transblotted histones incubated with radiolabeled C-terminal (56 kDa) half; in lanes 5 and 6 with radiolabeled N-terminal (64 kDa) half of ADPRT; in lanes 7 and 8 with radiolabeled ADPRT itself as described in Materials and Methods.

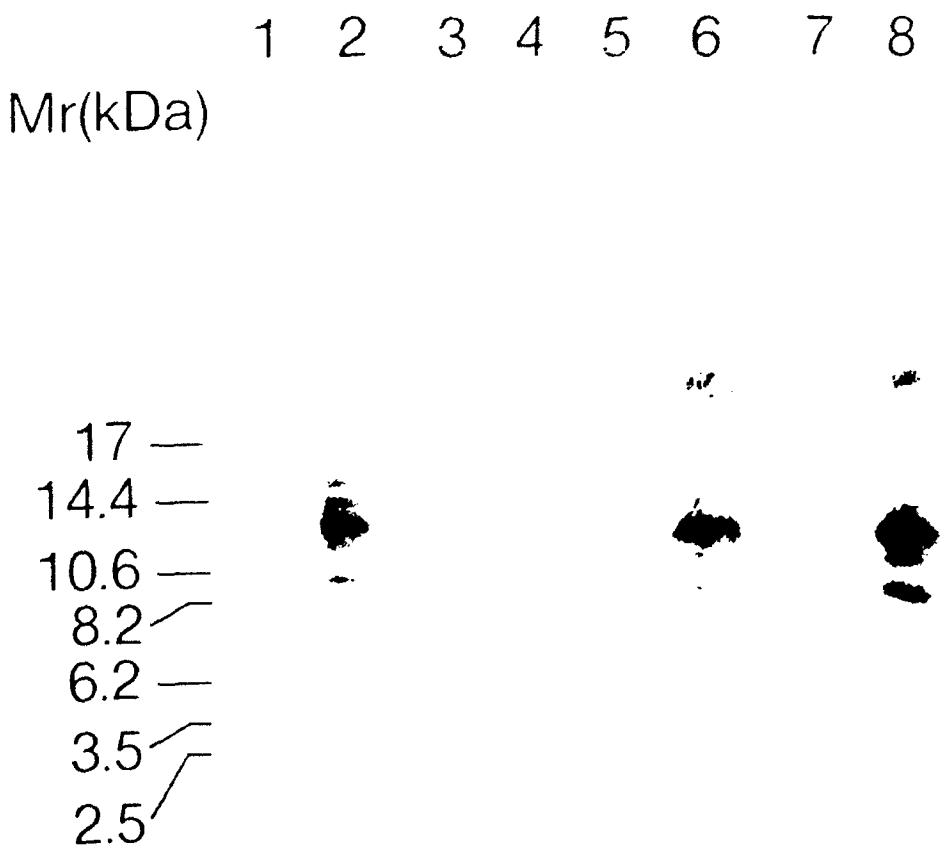

1 2 3 4 5 6 7 8
Mr(kDa)
17
14.4
10.6
8.2
6.2
3.5
2.5

Fig 4. Histones-Sepharose affinity chromatography of CNBr fragments of ADPRT.

Histones-Sepharose (1 ml bed volume) was loaded with 50 mg of unseparated CNBr fragments dissolved in 0.4 ml of 50 mM Tris-HCl, 10 mM 2-mercaptoethanol (pH 8) and was allowed to bind for 30 min. 25°C. Then the column was stepwise eluted with 2 ml aliquots of buffer containing increments of NaCl. The eluted fractions were concentrated on Centricon 3, one third of the total amount of the concentrates applied onto 17.5% SDS-PAGE. The gel was stained with Coomassie blue. Lane 1: unseparated CNBr-generated peptides; lane 2: peptides not bound to column; lanes 3-7 eluates with 50, 100, 200, 400, 1000 mM NaCl respectively.

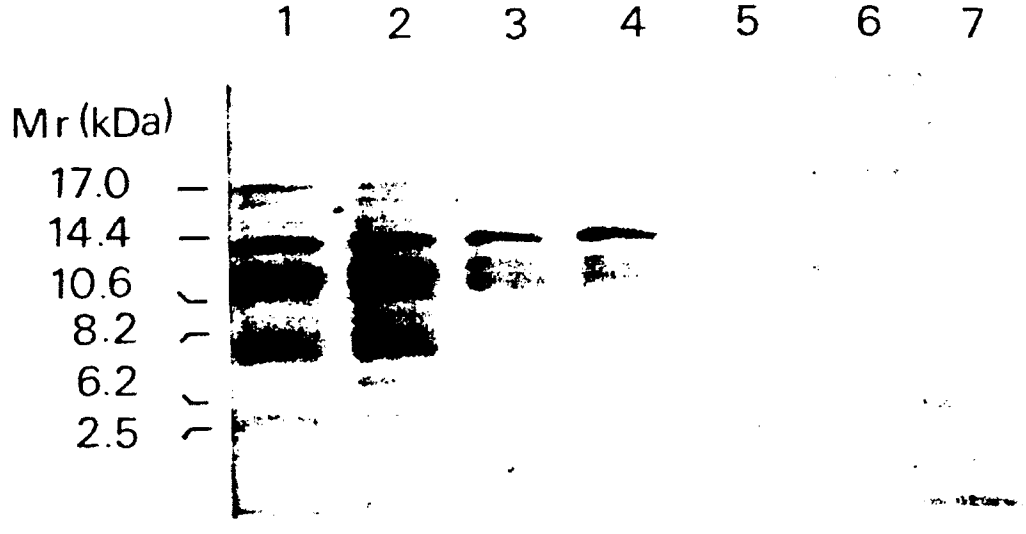
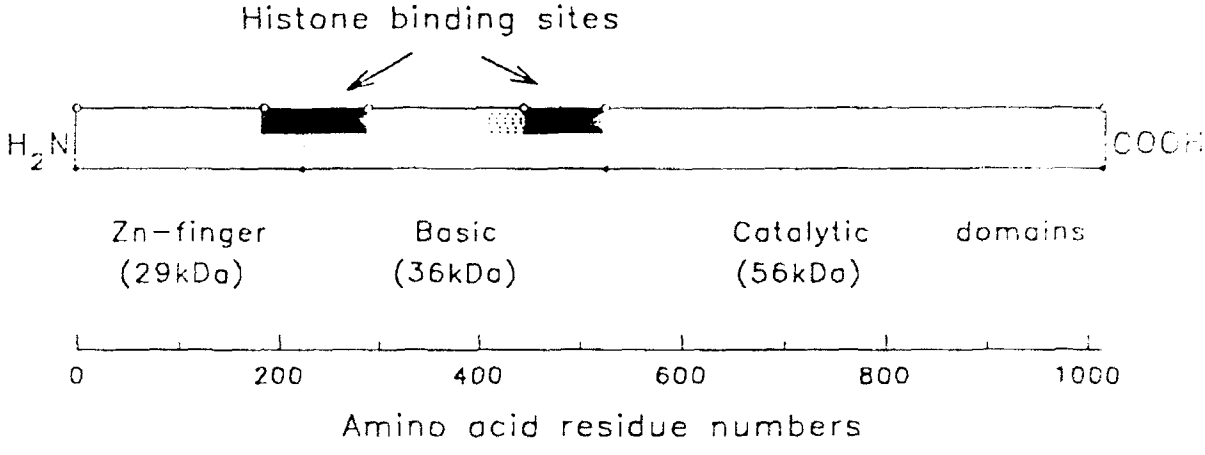

1
2
3
4
5
6
7
Mr (kDa)
17.0
14.4
10.6
8.2
6.2
2.5

Fig. 5. Location of histone-binding CNBr fragments on the primary sequence of ADPRT. Domains obtained by plasmin digestion (18) are also marked. The dotted area represents part of the 18 kDa fragment beyond the 11 kDa fragment.


Histone binding sites
H_2N
COOH
Zn−finger
(29kDa)
Basic
(36kDa)
Catalytic
(56kDa)
domains
0
200
400
600
800
1000
Amino acid residue numbers