Volume 17 Number 12 1989 Nucleic Acids Research

Poly(ADP-ribose)polymerase: a novel finger protein

Alice Mazen, Josiane Menissier-de Murcia, Miguel Molinete, Frédéric Simonin, Gérard Gradwohl, Guy Poirier[1] and Gilbert de Murcia*

IMBC du CNRS, Laboratoire de Biochimie 2, 15 rue René Descartes, 67084 Strasbourg Cedex, France and [1]Department of Molecular Endocrinology, Laval University Medical Center, 2705 Boulevard Laurier, Sainte Foy, Quebec GIV 4G2, Canada

Received January 17, 1989; Revised and Accepted May 11, 1989

ABSTRACT

By Energy Dispersive X-ray fluorescence we have determined that calf thymus poly(ADP-ribose) polymerase binds two zinc ions per enzyme molecule. Using ^{65}Zn (II) for detection of zinc binding proteins and polypeptides on western blots, we found that the zinc binding sites are localized in a 29 kd N-terminal fragment which is included in the DNA binding domain. Metal depletion and restoration experiments proved that zinc is essential for the binding of this fragment to DNA as tested by Southwestern assay. These results correlate with the existence of two putative zinc finger motifs present in the N-terminal part of the human enzyme. Poly(ADP-ribose)polymerase fingers could be involved in the recognition of DNA strand breaks and therefore in enzyme activation.

INTRODUCTION

Poly(ADP-ribose)polymerase is a chromatin-associated enzyme of eukaryotic cell nuclei, which catalyzes the covalent attachment of ADP-ribose units from the coenzyme NAD^+ to various nuclear acceptor proteins. This post-translational modification has been postulated to influence a number of chromatin functions, especially those involving nicking and resealing of DNA strands (1−5). In addition, modification by poly(ADP-ribosyl)ation of chromatin components, namely histones, was shown to affect *in vitro* chromatin structure in a reversible manner (6,7).

Poly(ADP-ribose)polymerase (116 Kd) is a multifunctional enzyme. It strictly requires DNA strand breaks for catalytic activity (8). Following limited proteolysis of the purified protein, Kameshita et al. (9) have identified three functional domains in the enzyme molecule: a 46 Kd fragment which act as the DNA binding domain and is located in the N-terminal region, a central 22 Kd polypeptide fragment containing the site of automodification and a C-terminal fragment (54 Kd) containing the NAD^+ binding domain.

The recent publication of the cDNA sequence encoding the human enzyme (10,11) has now brought the study of the interaction of the enzyme with DNA strand breaks to the molecular level. Having recently developed a simple technique for the detection of zinc-binding proteins and polypeptides (12) and given the fact that poly(ADP-ribose)polymerase is a zinc metalloenzyme (13), in the present work we have attempted to localize the zinc binding sites with respect to the DNA binding domain of the calf thymus enzyme. Radioactive zinc, ^{65}Zn (II), and [^{32}P]-labelled nick translated DNA were used alternatively to analyse Western blots loaded with proteolytic fragments of purified calf thymus poly(ADP-ribose)polymerase. The same blots were further immunostained with monoclonal antibodies. Using this combination of approaches we have demonstrated that ^{65}Zn (II) specifically binds to the N-terminal fragment (29 Kd) located in the DNA binding domain corresponding to the putative 'zinc finger' region of the human enzyme sequence. Metal

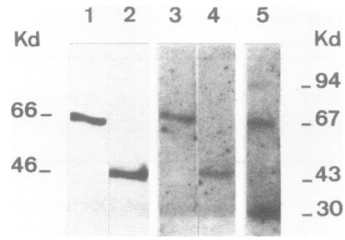

Figure 1: Zinc binding activity of the 46 Kd and the 66 Kd proteolytic fragments of poly(ADP-ribose)polymerase detected on western blots. Lanes 1 and 3 limited α-chymotrypsin digestion of the enzyme (7 μg) ; lanes 2 and 4 limited papain digestion of the enzyme (7 μg) ; lanes 1 and 2: immunostaining with the monoclonal antibody $C^{I}9$; lanes 3 and 4 : autoradiographs of the blots treated with ^{65}Zn (II) ; lane 5 low molecular weight standards labelled with ^{65}Zn(II) : 30 Kd carbonic anhydrase, 67 Kd bovine serum albumin.

depletion and restoration experiments proved clearly that zinc is essential for DNA binding to this portion of the protein. The methodology developed in the paper should be useful for the study of other 'zinc finger' proteins.

MATERIALS AND METHODS

MATERIALS

Biochemical reagents were purchased from Sigma, St Louis USA, unless otherwise noted. DNase I was purchased from Boehringer Mannheim Biochemicals, and DNA polymerase I from New-England Biolabs (Beverly MA). Molecular weight markers were purchased from Pharmacia. Nitrocellulose membrane filters (0.2 μm pore size) were obtained from Schleicher and Schuell.[α-^{32}P] dCTP was obtained from Amersham corporation and ^{65}Zn (II) from New- England Nuclear.

Calf thymus poly(ADP-ribose)polymerase was prepared according to the procedure of Zahradka and Ebisuzaki (13). Digestion of purified poly(ADP-ribose)polymerase with trypsin was perfomed at 20°C with a trypsin to protein ratio of 20:1. Limited digestion of the enzyme with papain was perfomed as described by Kameshita et al. (9). The reaction was terminated by adding trichloroacetic acid to a final concentration of 20%. The

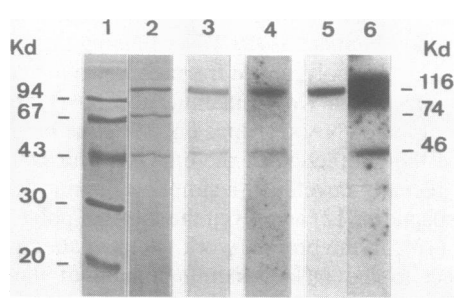

Figure 2: Zinc binding and DNA binding activities of whole enzyme and of papain digestion fragments of poly(ADP-ribose)polymerase. Stained gel : molecular weight markers (lane1) and papain digest (lane 2) resolved in 15% SDS gel. Lanes 3 to 6 : western blots of a papain digest (6 μg) immunostained with the monoclonal antibody $C^{I}9$ (lane 3), labelled with ^{65}Zn (II) (lane 4), incubated with ^{32}P nick-translated DNA and autoradiographed for 1 h (lane 5) or for 16 h (lane 6).
As an internal standard, the whole enzyme (1 μg) was added to each slot.

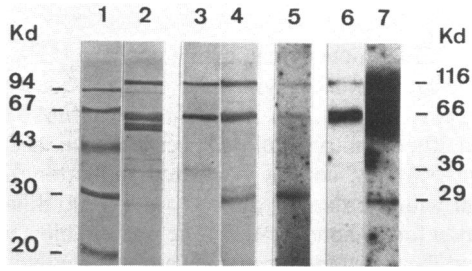

Figure 3: Co-localization of the zinc binding sites and the DNA binding domain on the 29 Kd N-terminal fragment of poly(ADP-ribose)polymerase. Stained gel : molecular weight markers (lane 1) and trypsin digest (lane 2). Lanes 3 to 7 : western blots of a trypsin digest (6 μg) immunostained with the monoclonal antibodies $C^{II}10$ (lane 3) and $C^{I}9$ (lane 4), labelled with ^{65}Zn (II) and autoradiographed for 20 h (lane 5), incubated with ^{32}P labelled DNA and autoradiographed for 1 h (lane 6) or 16 h (lane 7). As an internal standard, the whole enzyme (1 μg) was added to each slot.

proteolytic fragments were washed three times with ice cold ethylether and finally dried under vacuum.

Calf thymus DNA was digested with DNase I to an average length of 500 base pairs. After deproteinisation the nicked DNA was radioactively labelled by nick-translation to a specific activity of 107−108 cpm/μg using [α-^{32}P] dCTP.

METHODS

Determination of zinc by Energy dispersive X-ray fluorescence (EDXRF) spectrometry

For metal analysis, ultrapure water (MilliQ instrument Millipore Corp.) in which the concentration of free Zn (II) was lower than 5.10^{-9}M was used. All glass and plastic ware were rinsed with metal free water.

Before zinc determination the protein storage buffer was exchanged against buffer A (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl-fluoride (PMSF), by spin dialysis.

Poly(ADP-ribose)polymerase was concentrated in a Speedvac concentrator (Savant) to a final concentration of 1 mg/ml. Protein concentration was measured by the method of Bradford (14). An equivalent volume of buffer A was also concentrated under the same experimental conditions.

Metal analysis was performed by Energy Dispersive X-Ray Fluorescence (EDXRF) using a prototype spectrometer developed by the Siemens Company (Karlsruhe-FRG) in collaboration with the Laboratoire de chimie Minérale UA405 du CNRS, Strasbourg France (15).

SDS-PAGE electrophoresis and electrophoretic transfer

Poly(ADP-ribose)polymerase and its proteolytic fragments were separated on 15% polyacrylamide-SDS minigels (7 cm×7 cm) according to the protocol described by Laemmli (16). As an internal standard, the whole enzyme (1 μg) was added to each proteolytic digest in the sample buffer. For zinc binding experiments the gels were preincubated 1 hour at 37°C in the reduction buffer as described previously (12). Electrotransfer of proteins onto nitrocellulose sheets was performed according to Towbin et al. (17) at 4°C in a miniblot apparatus for 1 hour at 200 mA.

Blots of the enzyme and of proteolytic fragments were analysed with three different specific probes : monoclonal antibodies, ^{65}Zn (II) and [^{32}P] nick-translated DNA.

Binding experiments

(i) *Immunoreactivity* : The monoclonal antibodies C^{I}_{9}, C^{II}_{10}, C^{I}_{2} specific for the functional domains of the calf thymus enzyme have been previously characterized (18,19).

The electroblots were washed with TBS-tween buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.3% v/v Tween 20), the nitrocellulose sheet was then incubated for 1 hour in the same buffer containing 5% w/v non-fat powdered milk (blocking solution) at room temperature. Incubation with the desired antibody at 1:1000 dilution was performed in the same blocking solution for 16 hours at 4°C. The western blots were then washed twice in TBS-tween buffer and the transfered proteins were revealed with the use of antimouse alkaline phosphatase conjugated antibody (20).

(ii) *Metal binding* : The proteins and polypeptides transfered to nitrocellulose sheets were probed with ^{65}Zn (II) as previously described (12). Briefly, the blots were first incubated for 1 hour at 4°C in 50 ml of 10 mM Tris-HCl buffer pH 7.5. The strips were then soaked for 15 min in 5 ml metal binding buffer (10 mM Tris-HCl pH 7.5, 10 mM KCl) containing 1μCi $^{65}ZnCl_2$ and washed for a total of 30 min with two changes of binding buffer. The blots were dried and analyzed by autoradiography using X-AR film (Kodak).

(iii) *DNA binding* : (Southwestern blots). After transfer of the proteins onto nitocellulose sheets, the blots were washed for 30 min in TBS-Nonidet-P40 (NP40) buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% vol/vol NP40). Preincubation of the blots was performed during 30 min in the DNA binding buffer containing 20 mM Tris-HCl pH 8, Dithiothreitol (DTT) 2 mM, KCl 100 mM 0.1% v/v NP40). The blots were then incubated for 1 hour in a sealable plastic bag with 1 ml of binding buffer containing 20 ng of [^{32}P] labelled nick-translated DNA (107 − 108 cpm/μg). After 3 changes of the binding buffer, the nitrocellulose sheets were dried and autoradiographed using X-AR films. All incubations were at room temperature. Blots which had been probed with ^{65}Zn (II) or [^{32}P] DNA were also immunostained following autoradiography.

RESULTS

Metal content of poly(ADP-ribose)polymerase

Metal content of calf thymus poly(ADP-ribose)polymerase was determined by EDXRF spectometry (15). 20 μl of the concentrated enzyme solution (7.0 ± 0.5 μM) was employed for metal determination relative to two internal standards. The technique revealed no significant content of Fe (II) and Cu (II) in poly(ADP-ribose)polymerase but the presence of Zn (II) at a concentration of 15 ± 1 μM was detected giving a ratio of 2 ± 0.26 mole of zinc per mole of enzyme. No zinc could be detected in the concentrated buffer sample submitted with the protein sample to spectometry.

Zinc binding sites are located in the DNA-binding domain of poly(ADP-ribose)polymerase.

In order to localize the zinc atoms with respect to the different functional domains of the enzyme molecule, we have developed a simple and specific technique to detect zinc-binding proteins transfered to nitrocellulose and probed with ^{65}Zn (II) (12). This assay was used in this work to test on western blots the zinc binding capacity of the different polypeptides fragments generated by limited proteolysis of calf thymus poly(ADP-ribose)polymerase. Figure 1 shows that the 66Kd fragment as well as the 46 Kd fragment

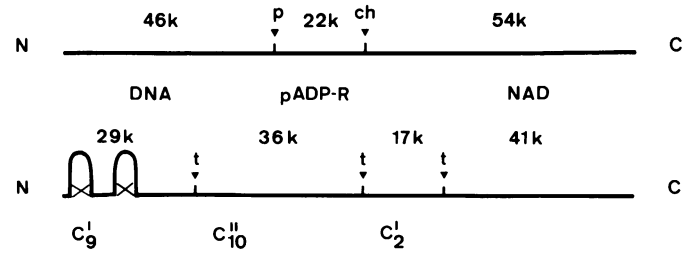

Figure 4: Schematic representation of the localization of the zinc binding sites (X) inside the DNA binding domain of poly(ADP-ribose)polymerase. The upper part represents the three different domains obtained after papain (p) and chymotrypsin (ch) digestion according to ref (9). The lower part represents the polypeptides obtained after trypsin digestion (t). The approximative position of the epitopes corresponding to the monoclonal antibodies $C^{I}9$, $C^{II}10$ and $C^{I}2$ is also indicated according to ref (19).

obtained by limited a chymotrypsin and papain proteolysis, respectively, bind ^{65}Zn (II) specifically (fig.1 slots 3−4). These two fragments of the enzyme have been reported to contain the DNA binding sites (9). The same blots already treated for zinc binding were also immunostained with the monoclonal antibody $C^{I}9$ reacted with the DNA binding domain of the calf thymus enzyme (18,19), thus demonstrating that the zinc binding sites are included within the domain recognized by $C^{I}9$, i.e. the DNA binding domain.

The ability of some fragments to bind both zinc and DNA was established directly using blots of the intact enzyme and of papain digestion fragments which were tested in parallel with three different specific probes : the monoclonal antibody $C^{I}9$, ^{65}Zn (II), and [^{32}P] nick translated DNA. Figure 2 displays a papain digest of the calf thymus enzyme (116 Kd) after transfer to nitrocellulose. It can be seen that only the undigested enzyme and the 46 Kd fragment both of which react with the $C^{I}9$ antibody, are also labelled with ^{65}Zn (II) and with [^{32}P] DNA. This result confirms the location of the zinc binding sites within the 46 Kd DNA binding domain.

Further dissection of the enzyme was carried out using trypsin digestion in order to determine the smallest fragment reacting simultaneously with ^{65}Zn (II) and [^{32}P] labelled DNA.Trypsin digestion generated first two fragments of 66 Kd and 54 Kd which where split upon prolonged digestion into two fragments of 29 and 36 Kd and 41 and 17 Kd respectively (fig. 3 slot 2). The identity of the tryptic fragments has been established by their reactivity with monoclonal antibodies $C^{I}9$ and $C^{II}10$ (18,19). Both were shown to react with the entire enzyme (116 Kd) and with the 66 Kd fragment, but the $C^{I}9$ antibody bound to the 29 Kd−N-terminal domain of the protein whereas the $C^{II}10$ antibody bound to the 36 Kd fragment (fig. 3 slots 4−3) which also possess the automodification sites (19). The autoradiography of the blots probed with ^{65}Zn (II) (fig. 3 slots 5) or with [^{32}P] labelled DNA (fig. 3 slot 6−7) revealed that both ligands bind to exactly the same fragments (116, 66 and 29 Kd) which react also with the $C^{I}9$ monoclonal antibody.

Taken together, these results prove that the zinc binding sites and the DNA binding sites are both located within the 29 Kd−N-terminal part of the protein, since no reactivity associated with other shorter fragments was observed in our experimental conditions. These conclusions are represented schematically in figure 4, which shows the approximate positions of the various proteolytic fragments, the location of the regions recognized by the specific

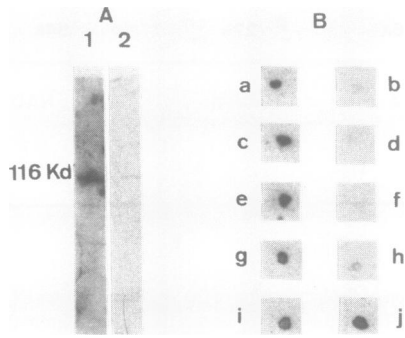

Figure 5: Exchangeability of ^{65}Zn (II) bound to poly(ADP-ribose) polymerase (116 Kd).
A : western blots of the enzyme (3 μg) incubated both with ^{65}Zn (II) ; in slot 2 the blotted enzyme was reincubated with 100 μM non radioactive zinc acetate under the same experimental conditions. B : dot blots of the enzyme (0.8 μg) incubatedwith ^{65}Zn (II) washed with metal binding buffer and reincubated under the same conditions either with 5mM EDTA (b) or with 100 μM Fe (II) (c) ; Zn (II) (d) ; Co (II) (e) ; Cu (II) (f) ; Mn (II) (g) ; Cd (II) (h) ; Ni (II) (i) ; Mg (II) (j) ; control (a).
The nitrocellulose filters were autoradiographed for 18 h.

probes and the positions of the epitopes recognized by the different monoclonal antibodies used in this study.

Metal replacement experiments

The ability of ^{65}Zn (II) bound to poly(ADP-ribose)polymerase to exchange with various other bivalent metal ions such as Zn (II), Co (II), Mn (II), Cd (II), Fe (II), Ni (II), Mg (II), and Cu (II) was carried out using EDTA as chelating agent.

As seen in figure 5A 1−2, radioactive zinc bound to poly (ADP- ribose)polymerase transfered nitro-cellulose sheets could be displaced by nonradioactive zinc acetate at a concentration of 100 μM in the metal binding buffer as shown in figure 5A slots 1−2.

Dot blots of the enzyme (116 Kd) spotted on nitrocellulose sheets were also used for metal exchange experiments and to test for zinc removal by 5 mM EDTA. After incubation

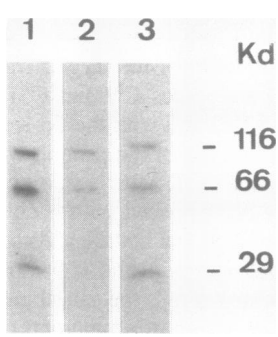

Figure 6: Role of zinc in the interaction between poly(ADP-ribose) polymerase and DNA.
A trypsin digest of the enzyme (3 μg) blotted onto nitrocellulose sheets was incubated with ^{32}P labelled DNA after either : (1) no further treatment of the blot (control) ; (2) addition of 5 mM EDTA in the washing buffer or (3) addition of 5mM EDTA in the washing buffer as in (2) followed by an incubation in the washing buffer containing 100 μM nonradioactive zinc acetate to restore the metal content of the enzyme.

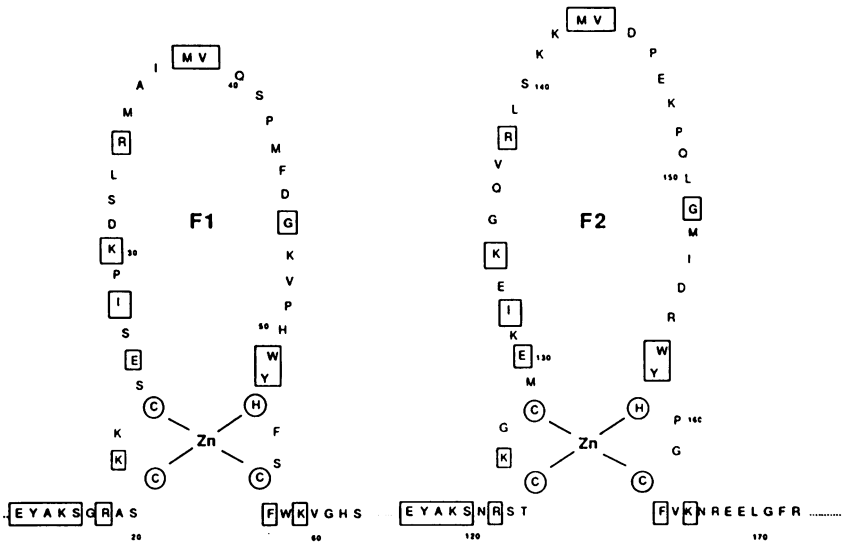

Figure 7: Schematic folding of the amino acid sequence of human poly(ADP-ribose)polymerase zinc fingers. Duplicated amino acids are boxed, according to ref. (10,11).

of the dot blots with ^{65}Zn (II) for 15 min and washing in metal binding buffer, a second incubation of the zinc blot was carried out for 15 min with 100 μM of the different divalent metal ions in the binding buffer. The results displayed in figure 5B show that ^{65}Zn (II) could be displaced by Zn (II) or by Cu (II) and partially exchanged by Cd (II) (fig. 5B, d,f,h, respectively).The other cations : Fe (II), Cd (II), Mn (II), Ni (II), and Mg (II) were unable to release the bound ^{65}Zn (II) (fig. 5B, c,e,g,i and j, respectively).

When 5mM EDTA was used in the washing step most of the radioactivity due to ^{65}Zn (II) binding was lost as shown in figure 5B-b thus demonstrating that Zn (II) ions could not only be exchanged but also released from the protein transfered to nitrocellulose.

The N-terminal 29 Kd fragment requires zinc for DNA binding

To assess the role of zinc in the binding of DNA we took advantage of the possibility to remove the metal ion from the blotted protein by the addition of 5 mM EDTA during the washing procedure. A mixture of 116, 66 and 29 Kd fragments was electrophoresed in triplicate on a 12% SDS gel and subsequently transfered to nitrocellulose.The western blot was then incubated with [^{32}P] labelled DNA as described above after either (i) no futher treatment of the blot (control, fig. 6 slot 1) or (ii) addition of 5 mM EDTA in the washing buffer for 30 min (fig. 6 slot 2) or (iii) addition of 5 mM EDTA as in (ii) followed by another 30 min incubation in the washing buffer containing 100 μM non radioactive zinc acetate to restore the metal content of the enzyme (fig. 6 slot 3). One can see that zinc removal causes an important decrease in the DNA binding capacity of the 116 and 66 Kd fragments and a complete disappearance of the radioactivity due to DNA binding to the 29 Kd fragment. Interestingly the DNA binding property of this fragment could be fully restored when 100 μM non radioactive zinc acetate was added to the binding buffer, whereas the signal corresponding to the 116 and 66 Kd peptides was slightly increased (fig. 6 slot 3) under the same experimental conditions.

DISCUSSION

Using EXAFS (extended X-ray absorption fine structure) spectroscopy, Diakun et al (35) have studied the ligands involved in co-ordination of the zinc atom in TFIIIA. In the present report we have used another recently developed spectrometry technique (EDXRF) (15) which allows multielemental determination of biological samples, in μmolar concentration and in small volume, to reinvestigate qualitatively and quantitatively the metal content of purified poly(ADP-ribose) polymerase. Two zinc atoms per enzyme molecule were found, a ratio which is about twice the value obtained by Zaradka and Ebisuzaki in their original work (13).

In order to map the metal binding sites with respect to the different functional domains of the enzyme we have recently developed a simple technique which allows the detection of zinc binding proteins transfered to nitrocellulose (12). Using ^{65}Zn (II) as a probe we found that the radioactivity specifically associated with the 116 Kd whole enzyme and with the 66 and the 46 Kd fragments generated by a chymotrypsin and papain respectively. These two fragments where shown to contain DNA binding sites (9). A 29 Kd fragment obtained by trypsin digestion of the enzyme was found to be the shortest polypeptide still having the capacity to bind ^{65}Zn (II). The assignment of this fragment to the N-terminal part of the protein was facilited by the use of monoclonal antibodies (18,19).

By Southwestern blotting performed on the same proteolytic fragments, we have shown that the zinc binding sites are totally included in the DNA binding domain, but not in the catalytic part of the protein like other NAD enzymes such as dehydrogenases (21). A similar approach was performed for the determination of the zinc and double-stranded RNA binding sites in the Reovirus outer capsid protein σ3 (34).

Metal exchange experiments demonstrated that ^{65}Zn (II) could be chased by nonradioactive zinc acetate and could be totally or partially displaced by Cu (II) or Cd (II) respectively. Using EDTA as a metal chelator we found that DNA binding detected on the 29 Kd fragment was inhibited by zinc depletion and subsequently restored by adding back the metal to the transfered protein. However the DNA binding observed for the 116 Kd enzyme as well as the 66 Kd polypeptide after EDTA treatment suggest the existence of the second DNA binding site, zinc independent, which could be located in the c-terminal part of the 46 Kd fragment. Indeed Buki and Kun (22) using plasmin degradation, recently detected a 36 Kd fragment located between the N-terminal 29 Kd fragment and the 54 Kd NAD binding domain. This 36 Kd polypeptide exhibited DNA binding properties.

A novel protein motif called a 'zinc finger' capable of nucleic acid binding was first discovered in the Xenopus transcription factor IIIA (23) but now appears to be ubiquitous (24,25). In TFIIIA nine independent domains are stabilized by 7,11 zinc ions in the ribonucleoprotein complex, each repeat unit binding a zinc metal ion through invariant Cys and His residues involved in the consensus sequence : (Cys/His)$-X_{aa\ 2-4}-$(Cys/His)$-X_{aa\ 2-15}-$(His/Cys) $X_{aa\ 2-4}-$(His/Cys).These residues are thought to form a tetrahedral complex with the metal ion such that the central $X_{aa\ 2-15}$ residues are organised in a loop or finger structure interacting with DNA. Examination of the recently published human poly(ADP-ribose)polymerase sequence deduced from the cloned cDNA (10,11) reveals two repeated putative zinc finger motifs in the N-terminal part of the DNA binding domain at position 2$-$97 and 106$-$207. The remarkable degree of conservation of the primary structure observed when one compare the partial sequences known for the calf thymus enzyme (22,26) to the full length human enzyme makes it likely that two zinc finger motifs exist in the N-terminal 29 Kd fragment of the bovine poly(ADP-

ribose)polymerase also. This in turn would correlate perfectly with the presence of the two zinc ions we have detected by EDXRF, and which would be implicated in the proper folding of this polypeptide for DNA binding. A schematic model of this structure is proposed in figure 7.

Although the term 'zinc finger' protein was initially restricted to regulatory proteins having finger motifs closely related to TFIIIA (27) this family has now broadened its scope by the recent recruitment of the nuclear receptors (28–30) and the nucleic acid binding motifs associated with the retroviral gag gene protein (31) and T4 phage gene 32 protein (32,33). On the basis of our results we propose that poly(ADP-ribose)polymerase should also be considered as a member of the zinc-finger protein family. Given the fact that the enzyme activity is triggered by DNA strand breaks (8) it is tempting to speculate that in this case the finger domain of the protein could be involved in the recognition of DNA interruptions.

ACKNOWLEDGEMENTS

We thank Dr E. Maier and Dr M. Leroy for EDXRF measurements, we thank also Josette Dunand for excellent technical assistance and Dr. K. Richards for critical revision of the manuscript.

This work was carried out in Prof. Dirheimer's laboratory and was supported by the Ligue National Française contre le Cancer (Delegation Departementale du Haut-Rhin)

G. G. was supported in part by a predoctoral fellowship from Association pour la Recherche contre le Cancer ARC.

*To whom correspondence should be addressed

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