Photoaffinity labelling of human poly(ADP-ribose) polymerase catalytic domain

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Photoaffinity labelling of the human poly(ADP-ribose) polymerase (PARP) catalytic domain (40 kDa) with the NAD⁺ photoaffinity analogue 2-azido-[α -³²P]NAD⁺ has been used to identify NAD⁺-binding residues. In the presence of UV, photoinsertion of the analogue was observed with a stoichiometry of 0.73 mol of 2-azido-[α -³²P]NAD⁺ per mol of catalytic domain. Competition experiments indicated that 3-aminobenzamide strongly protected the insertion site. Residues binding the adenine ring of NAD⁺ were identified by trypsin digestion and boronate affinity chromatography in combination with reverse-phase

HPLC. Two major NAD⁺-binding residues, Trp¹⁰¹⁴ of peptide Thr¹⁰¹¹–Trp¹⁰¹⁴ and Lys⁸⁹³ of peptide Ile⁸⁷⁹–Lys⁸⁹³, were identified. The site-directed mutagenesis of these two residues revealed that Lys⁸⁹³, but not Trp¹⁰¹⁴, is critical for activity. The close positioning of Lys⁸⁹³ near the adenine ring of NAD⁺ has been confirmed by the recently solved crystallographic structure of the chicken PARP catalytic domain [Ruf, Ménissier-de Murcia, de Murcia and Schulz (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 7481–7485].

INTRODUCTION

In eukaryotes, DNA-strand breaks, introduced either directly by ionizing radiation or indirectly after enzymic incision of a DNA lesion, trigger the synthesis of poly(ADP-ribose) by the enzyme poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30). At a site of DNA breakage, PARP catalyses the transfer of the ADP-ribose moiety from the respiratory coenzyme NAD⁺, to a limited number of protein acceptors involved in chromatin architecture or in DNA metabolism, including the enzyme itself. These modified proteins, carrying long chains of negatively charged ADP-ribose polymers, have a decreased affinity for DNA, which alters their function [1–3]. The short half-life of this polyanion is attributed to the high activity of poly(ADP-ribose) glycohydrolase (PARG), which cleaves the ribose–ribose bond that links ADP-ribose residues [4]. Therefore poly(ADP-ribosylation) is an immediate but transient post-translational modification of nuclear proteins induced by DNA-damaging agents.

Poly(ADP-ribosylation) reactions are believed to be involved in the regulation of strand-break rejoining and in cell recovery from DNA damage. Although its precise biological role has not been fully elucidated, PARP has emerged in the past decade as a critical regulatory component of the immediate cellular response to DNA damage and particularly in the base excision repair pathway [5–7].

PARP is a multifunctional, highly conserved enzyme that binds tightly to DNA breaks, stabilizing a V-shaped DNA conformation [8]. The protein (113 kDa) has a modular organization. A 46 kDa N-terminal DNA-binding domain acts as a

molecular nick sensor and contains two zinc-finger motifs [9,10] and a bipartite nuclear location signal [11]. A 22 kDa central region, containing auto-poly(ADP-ribosylation) sites, presumably regulates PARP–DNA interactions. The most highly conserved domain is the 54 kDa C-terminal catalytic domain, which contains all the catalytic activities associated with the full-length enzyme: abortive NAD⁺ hydrolysis, initiation, elongation, branching and termination of polymer synthesis. The catalytic activity can be further restricted to a 40 kDa domain without loss of enzymic activity [12], although the isolated domain contains a basal activity independent of the presence of DNA breaks. The K_m for NAD⁺ is comparable with that of the full-length PARP (50 μ M), but its specific activity is approximately 1/500 of that of the whole enzyme fully activated by DNA strand breaks [13]. The high specific activity of PARP is absolutely dependent on binding to DNA strand breaks and hence on the integrity of the zinc-finger domains [10,14].

To gain further insight into the structure and the function of the catalytic domain, in the present study we have used the photoaffinity probe 2-azido-[α -³²P]NAD⁺ to label the catalytic domain of the human PARP. After photolabelling, the photo-labelled peptides were purified by boronate chromatography in combination with reverse-phase HPLC. Two major NAD⁺-binding residues, Trp¹⁰¹⁴ and Lys⁸⁹³, were identified. The site-directed mutagenesis of these two residues revealed that Lys⁸⁹³, but not Trp¹⁰¹⁴, is critical for activity. The close positioning of Lys⁸⁹³ near the adenine ring of NAD⁺ has been confirmed by the recently solved crystallographic structure of the chicken PARP catalytic domain [15].

Abbreviations used: DT, diphtheria toxin; ETA, *Pseudomonas aeruginosa* exotoxin A; PARP, poly(ADP-ribose) polymerase; PTH, phenylthiohydantoin.

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EXPERIMENTAL

Materials

Biochemical reagents were, unless otherwise noted, purchased from Sigma (St. Louis, MO, U.S.A.). Nitrocellulose membrane (BAS 83) was obtained from Schleicher and Schuell (Dassel, Germany). [α - 32 P]NAD⁺ was obtained from NEN Corp. Molecular mass markers were purchased from Pharmacia LKB Biotechnology. pTG161 expression vector and *Escherichia coli* TGE 900 strain were obtained from Transgène (Strasbourg, France). The C-terminal domain of human PARP overproduced in *E. coli* was purified as previously described [13].

Characterization of photolabelling of the catalytic domain

2-Azido-NAD⁺ and 2-azido- $[\alpha$ - 32 P]NAD⁺ were synthesized as previously described [16]. To examine the properties of photolabelling, the 40 kDa PARP catalytic domain (approx. 1.0 μ g) was incubated with different concentrations of 2-azido- $[\alpha$ - 32 P]NAD⁺ in 10 μ l of 20 mM Hepes buffer, pH 7.2, on ice for 1 min before UV treatment. The specific radioactivity of the 2-azido- $[\alpha$ - 32 P]NAD⁺ was varied in different experiments by mixing radiolabelled and unlabelled preparations. Samples were irradiated on ice with a Spectroline medium-wavelength UV lamp (6.4 mW/cm²). Reactions were quenched by the addition of an equal volume of a protein-solubilizing mixture consisting of 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M Tris/HCl, pH 6.8, 1% (v/v) 2-mercaptoethanol and 0.002% Bromophenol Blue. Samples were analysed by SDS/PAGE with 10% gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue, destained, dried on a slab gel dryer and subjected to autoradiography. For quantitative analysis of binding of radiolabelled 2-azido-NAD⁺ to the enzyme, the stained protein bands were excised and radioactivity was determined by liquid-scintillation counting. To determine the effect of UV irradiation on the activity of the catalytic domain, incubations containing 50 μ M unlabelled 2-azido-NAD⁺ were diluted after photolabelling and enzyme activity was determined with $[\alpha$ - 32 P]NAD⁺ as described previously [17].

Table 1 Nucleotide sequences of reverse primers used in PCR experiments

The *Xba* I site introduced is shown in bold. Changed nucleotides are underlined. Deletions are represented by ^. The stop codon is in italics.

Mutation*	Nucleotide sequence
W1014A	gaat cttaga TTACGQCAGGGAGGTCTTAA AA TT
L1013A	gaat cttaga TTACCACGCGGAGGTCTTAA AA TT
S1012A	gaat cttaga TTACCACAGGGCGGTCTTAA AA TT
T1011A	gaat cttaga TTACCACAGGGAGGCTTAA AA TT
K1010A	gaat cttaga TTACCACAGGGAGGTGCA AA TT
F1009C	gaat cttaga TTACCACAGGGAGGTCTTGC AA TT
N1008A	gaat cttaga TTACCACAGGGAGGTCTTAA AA AGCGAA
F1007A	gaat cttaga TTACCACAGGGAGGTCTTAA AA ATTGCGTTT
Δ 1007-1014	gaat cttaga TTA [^] TTTCAGTTTCAGCAGATA
Δ 1011-1014	gaat cttaga TTA [^] CTTAA AA TTGAATTTCAG

* The first letter is the one-letter code for the wild-type amino acid followed by its position in the human sequence; the second letter indicates the mutated amino acid. Δ indicates that the numbered residues were deleted.

Isolation of photolabelled peptides

For preparative photolabelling, the catalytic domain (400 μ g) was photolabelled for 1 min with 200 μ M 2-azido- $[\alpha$ - 32 P]NAD⁺ in a final volume of 2.0 ml as described above. Protein was precipitated by adding an equal volume of ice-cold 7% (w/v) perchloric acid to remove excess unbound ligand. The precipitate was washed once with ice-cold 3.5% perchloric acid and once with ice-cold acetone. The protein was resuspended in 0.5 ml of 0.1 M NH₄HCO₃ buffer, pH 8.0, containing 2 M urea, and digested with 5.0% (w/w) 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated trypsin at 30 °C overnight. The tryptic digest was diluted to 2.0 ml with 0.1 M ammonium acetate buffer, pH 8.9 (buffer A), and applied to a 1.0 ml column of Amicon PBA-30 equilibrated with buffer A. The column was washed with 12 ml of buffer A to remove unmodified peptides. The photolabelled peptides were eluted with buffer A containing 0.1 M sorbitol. Fractions of 1.0 ml were collected and radioactivity was determined by liquid-scintillation counting. The photolabelled peptides purified by boronate affinity chromatography were fractionated by reverse-phase HPLC on an Aquapore C₄ guard column (2.1 mm $\times$ 30 mm). The peptide fraction was adjusted to 0.1% in trifluoroacetic acid and applied to the column followed by elution with 0.1% trifluoroacetic acid containing a linear gradient of 0–40% acetonitrile, increasing at 0.5% per min. The UV absorbance of the eluted peptides was monitored by a diode-array spectral detector. Fractions of 0.5 ml were collected and radioactivity was determined by liquid-scintillation counting.

Peptide sequencing

Photolabelled peptides were sequenced by automatic Edman degradation, with an Applied Biosystems gas-phase sequencer model 470A, equipped with a phenylthiohydantoin amino acid (PTH aa) analyser model 120A [18]. Radioactivity released during each Edman degradation cycle was measured by liquid-scintillation counting.

Site-directed mutagenesis of the catalytic domain of human PARP

The prokaryotic expression vector pTG161 PARP expressing the full-length human PARP has been previously described [12,13]. The substitutions and deletion mutations were introduced by means of PCR with appropriate synthetic oligonucleotides as primers. Reactions were done in a volume of 100 μ l consisting of 200 μ M each dNTP, 20 mM Tris/HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 1 μ g of the sense primer (nucleotide sequence TTCTGAAGAAGC-CGA), 1 μ g of reverse primers (see Table 1 for nucleotide sequences), 12.5 ng of the pTG161 PARP plasmid and 2 units of *Vent* polymerase. Amplification was performed (25 cycles; 95 °C, 2 min; 50 °C, 1 min; 70 °C, 1 min). The amplified DNA was then cloned back into the pTG161 PARP plasmid by replacing the *EcoRV*-*Xba*I fragment with the mutated one. The DNA sequences were determined by the dideoxynucleotide chain termination method [19] to verify that the desired changes had been obtained.

Production and analysis of recombinant proteins

The production and analysis of the overproduced proteins have been described previously [13,20,21]. Briefly, the expression constructs were used to transform *E. coli* TGE900 cells. Typically, 1.5 ml of culture of plasmid-containing TGE900 cells was grown and heat-induced [22]. Bacteria were centrifuged and resuspended in 300 μ l of 25 mM Tris/HCl, pH 8.0, 50 mM glucose, 10 mM EDTA and 150 μ l of sample buffer [50 mM Tris/HCl (pH 6.8)/6 M urea/6% (v/v) 2-mercaptoethanol/3% (w/v)

SDS/0.003 % Bromophenol Blue]. Cells were then sonicated (one 60 s pulse at 180 V) and subjected to SDS/PAGE [10 % (w/v) gel] [23]. Immunoreactivity and activity blotting experiments were performed as described previously [24,25].

Enzymic assay and analysis of kinetic parameters

To estimate the amounts of wild-type and mutant PARP proteins a standard curve was generated by immunoblotting, with purified human PARP and a polyclonal antibody directed against the second zinc-finger of human PARP [25]. The intensities of the immunoreactive bands were quantified by densitometry [26]. Protein samples were incubated in the standard enzymic assay in solution with $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ (0.1 mCi/nmol). Kinetic analysis of wild-type and mutated PARP was performed with the partly purified protein extracts; K_m and k_{cat} values were calculated from triplicate experiments by using Lineweaver–Burk plots.

RESULTS

Criteria for specific photoaffinity labelling of the catalytic domain by 2-azido- NAD^+

2-Azido- NAD^+ has previously been shown to serve as a substrate for PARP [27], indicating that it should be useful for photolabelling of the active site of the enzyme. To determine whether this photoprobe would stably modify the catalytic domain, the enzyme was incubated with 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ and analysed by SDS/PAGE. Figure 1 (top panel) shows that the protein was labelled in a UV-dependent manner. The lack of labelling in the absence of UV irradiation eliminated the possibility that labelling was due to the enzymic activity of the catalytic domain.

To establish that the observed photolabelling of the catalytic domain represented modification specifically at or near the NAD^+ -binding site of the enzyme, a series of experiments were performed to test several criteria for specific photolabelling. Specific modification should be both time- and dose-dependent. Figure 1 shows that these two criteria were met, as photoinsertion of 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ into the catalytic domain was saturated after 20 s of UV treatment (Figure 1, middle panel) and, with a 30 s period of irradiation, labelling was saturated at 50 μM 2-azido- NAD^+ (Figure 1, bottom panel). To determine the stoichiometry of the modification, protein bands were excised from the gels and the amount of radiolabelled nucleotide was determined. At saturation, 12.5 pmol of protein bound 9.5 pmol of photoprobe, indicating a stoichiometry of 0.76 mol of photoprobe bound per mol of protein. When reaction mixtures were analysed by a filter-binding assay, a stoichiometry of 0.70 mol/mol was estimated. Because the catalytic domain contains a single NAD^+ -binding site, the stoichiometry of modification was also consistent with specific modification.

Another criterion for modification at the active site was met by the observation that photoincorporation of 2-azido- NAD^+ irreversibly inhibited the activity of the enzyme. UV irradiation in the absence of 2-azido- NAD^+ did not significantly affect the enzyme activity, whereas more than 90 % of the activity was lost in a time-dependent manner in the presence of 2-azido- NAD^+ after UV irradiation (results not shown). Finally, the specificity of the photolabelling was examined in studies that examined the ability of compounds to compete with 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ for photolabelling of the catalytic domain. Figure 2 shows that 3-aminobenzamide, which effectively competes for the nicotinamide moiety of NAD^+ for binding to PARP [1], inhibited photolabelling by 50 μM 2-azido- NAD^+ with an IC_{50} of 7–8 μM . As expected, unlabelled 2-azido- NAD^+ also provided good protection from photolabelling by 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$. Unex-

Figure 1 SDS/PAGE analysis of PARP catalytic domain incubated with 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ (top panel), and time dependence (middle panel) and concentration dependence (bottom panel) of photolabelling of the catalytic domain by 2-azido NAD^+

Top panel: the catalytic domain of the enzyme was incubated with radiolabelled 2-azido- NAD^+ with (lanes b and d) or without (lanes c and e) UV irradiation. After incubation, samples were subjected to SDS/PAGE and autoradiography. Lanes a, b and c show Coomassie Blue staining, with molecular mass markers (lane a) identified at the left. Lanes d and e show the autoradiogram. Middle and bottom panels: after incubation, samples were subjected to SDS/PAGE, the protein bands were excised and the amount of radiolabel incorporated was determined by liquid-scintillation counting. The samples in the bottom panel were irradiated for 30 s.

pectedly, $\beta\text{-NAD}^+$ was a relatively poor competitor for photolabelling, but $\beta\text{-NAD}^+$ displayed a significantly better competition than $\alpha\text{-NAD}^+$ under the conditions of photolabelling. The poor competition of $\beta\text{-NAD}^+$ with 2-azido- NAD^+ can be explained by a slower off rate for 2-azido- NAD^+ than for unsubstituted NAD^+ . Taken together, these results indicate that the photolabelling by 2-azido- NAD^+ is specific for the active site of the catalytic domain of the enzyme.

Identification of photolabelled peptides

To identify the site(s) of the photolabelling, proteolytic fragments of the catalytic domain were generated by digestion with trypsin. Purification of photolabelled peptides can be greatly facilitated

Figure 2 Effect of competitors on photolabelling of catalytic domain by 2-azido-NAD⁺

Aliquots of catalytic domain were incubated for 30 s with 50 μM 2-azido-[α-³²P]NAD⁺ in the presence of the indicated concentrations of 3-aminobenzamide (○), unlabelled 2-azido-NAD⁺ (●), β-NAD⁺ (△), or α-NAD⁺ (▲). The amount of radiolabel incorporated into the enzyme was determined as described in the legend to Figure 1.

Figure 3 Separation of photolabelled peptides by HPLC

The catalytic domain was photolabelled with 2-azido-[α-³²P]NAD⁺ and subjected to protease digestion, then photolabelled peptides were isolated by boronate chromatography as described in the Experimental section. The material eluted from the boronate column was subjected to reverse-phase HPLC, fractions were collected and radiolabel was determined by scintillation counting.

by the use of boronate affinity chromatography [16]. This procedure takes advantage of the existence of *cis*-diols on the photolabelled peptides, allowing them to be conveniently separated from a large excess of unmodified peptides. The photolabelled peptides retained on the boronate resin were effectively eluted by 0.1 M sorbitol. The eluted radioactive peptides were further fractionated by reverse-phase HPLC (Figure 3). The flow-through fraction contained some residual unbound radiolabel and decomposed photoprobe. Three main peaks containing radiolabel were observed that were eluted at approx. 30, 42 and 52 min. These three peaks, designated 1, 2 and 3 respectively, also contained material with absorbance at 215 nm (results not shown). Spectral analysis of each peak showed an absorbance maximum at 264 nm, typical of peptides photolabelled by 2-azido-NAD⁺ as reported previously [16]. On the basis of radiolabel, peaks 1, 2 and 3 contained 818, 222 and 219 pmol of peptide respectively, corresponding to 8.2%, 2.2% and 2.2% of the total radioactivity initially present in the acid-insoluble pellet after photolabelling. Although the radiolabel not bound to the

Figure 4 Microsequencing of the photolabelled peptides: localization of the photoproducts within the tryptic fragments isolated by boronate chromatography

The major radiolabelled peptides were sequenced by automatic Edman degradation. Radioactivity released during each cycle of degradation was measured by liquid-scintillation counting.

protein probably represents photoprobe and/or decomposed photoprobe trapped in the acid-insoluble pellet, the possibility that a portion of the photoprobe bound to the protein was released by the sample preparation cannot be ruled out.

Peptide sequencing

Peptide 1: TSLW (residues 1011–1014)

This peptide corresponds to the four C-terminal residues of the protein. The first three amino acid residues (TSL) were unambiguously identified in the first three sequencing cycles (Figure 4). At cycle 4, neither radiolabel nor phenylthiohydantoin (PTH)-amino acid was detected. Although this method of sequence

Table 2 Kinetic parameters of wild-type and mutated PARP proteins

PARP activity was determined by scintillation counting of the [α - 32 P]NAD⁺ incorporated into acid-insoluble precipitates (see the Experimental section). Results are means $\pm$ S.D. for 3–4 determinations. Abbreviation: n.d., not determined.

Enzyme form	K_m (μ M)	k_{cat} (s^{-1})	Reference
Wild-type	50 $\pm$ 5	0.48	This study
K893R	59 $\pm$ 4	0.19	[21]
K893I	n.d.	< 0.001	[21]
W1014A	52 $\pm$ 5	0.64	This study

determination can result in partial loss of the C-terminal amino acid, PTH-Trp as the C-terminal amino acid should have been easily detected considering the high recovery of PTH-Leu in the preceding cycle. The absence of detectable PTH-Trp indicated that the C-terminal Trp residue had undergone modification by the photoprobe. Most of the radiolabel was recovered in the fifth Edman degradation cycle, although no PTH-Trp was detected in this cycle. These results suggested the following: (1) the site of photoinjection was not located in the peptide backbone, because modification at Leu-Trp would have prevented the high yield of PTH-Leu observed at cycle 3 [28]; (2) it seems unlikely that the nitrene formed by photoactivation of the azide of the photoprobe formed an N–O covalent bond with Trp at the C-terminus, because this modification would be acid-labile and would have resulted in loss of radiolabel before isolation of the labelled peptides [29]; (3) the most probable site of photolabelling was the indole ring of tryptophan. The high polarity of the photoprobe probably resulted in its retention on the glass fibre filter for an additional sequencing cycle. Indeed, amino acids with polar side chains are often released from the filters over two sequencing cycles. Taken together, the results indicate that, in the case of the peptide TSLW, the photolabelling occurred at the indole ring of the C-terminal Trp residue.

Peptide 2: IAPPEAPVTGYMFGK (residues 879–893)

The sequence of this tryptic peptide could be unambiguously established. Approx. 10% of the total radiolabel was released during the first two sequencing cycles (Figure 4), suggesting some acid hydrolysis of the photolabelled peptide during the washing treatment with trifluoroacetic acid before the sequencing cycles. We postulate that, owing to its high polarity, the photoprobe generated from NAD⁺ was not removed by the initial acid washes but was extracted from the filter by the first two sequencing cycles. This suggested an N–O covalent bond between the photoprobe and the peptide. Approx. 90% of the radiolabel was recovered in sequencing cycles 15 and 16, with a normal yield of PTH-Lys in cycle 15. Taken together, these results suggest the following: (1) the site of photolabelling does not seem to be at the peptide bond Gly¹⁴–Lys¹⁵ because both the amino acids were recovered during sequencing; (2) the side chain of Lys¹⁵ seems to be an unlikely site of the photolabelling because the modification of the ϵ -amino group of Lys would inhibit the cleavage by trypsin; (3) the most likely site of photolabelling is the oxygen of the carbonyl linkage of Lys to form an N–O covalent bond. The formation of such a derivative would explain the acid lability observed and the complete recovery of PTH-Lys at step 15. Additionally, such a modification would not affect the cleavage at Lys by trypsin. Thus we conclude that the photolabelling of this peptide most probably takes place at Lys⁸⁹³.

A	Hum	1000	K	Y	L	L	K	L	K	F	N	F	K	T	S	.	L	W	.	.	1014
	Bov	1002	K	Y	L	L	K	L	K	F	N	F	K	T	S	.	L	W	.	.	1016
	Rat		K	Y	L	L	K	L	K	F	N	F	K	T	S	.	L	W	.	.	
	Mou	999	K	Y	L	L	K	L	K	F	N	F	K	T	S	.	L	W	.	.	1013
	Chi	997	K	Y	L	L	K	L	K	F	N	F	K	T	S	.	L	W	.	.	1011
	Xen	996	K	Y	L	L	K	L	K	F	N	F	K	T	S	.	L	W	.	.	1011
	Den	983	q	Y	L	f	R	M	e	F	k	Y	.	.	.	.	s	Y	.	.	994
	Sar	986	q	Y	M	L	R	M	n	F	k	Y	K	.	.	.	.	.	.	.	996
	C.el	521	K	Y	V	V	e	V	K	V	D	r	l	T	A	k	e	m	a	.	538
	Ara	626	R	Y	V	I	q	V	K	F	N	Y	K	h	.	.	.	.	.	.	637
	Dic	901	K	Y	L	L	q	L	R	n	.	.	.	.	.	.	.	.	.	.	908

		PARP activity																	
B	wild-type	1000	K	Y	L	L	K	L	K	F	N	F	K	T	S	L	W	1014	+
	W1014A		.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	+
	L1013A		.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	+
	S1012A		.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	+	
	T1011A		.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	+	
	K1010A		.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	+	
	F1009C		.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	+	
	N1008A		.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	+	
	F1007A		.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	+	
	Δ 1007-1014		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	
	Δ 1011-1014		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	

Figure 5 Multiple alignment of the sequence of the C-terminal part of PARP from different species (A), and mutational analysis of the human PARP C-terminal region (B)

(A) Sequences are shown for the C-terminal part of PARP from human (Hum), bovine (Bov), rat (Rat), mouse (Mou), Chicken (Chi), *Xenopus laevis* (Xen), *Drosophila melanogaster* (Dro), *Sarcophaga peregrina* (Sar), *Caenorhabditis elegans* (C.el), *Arabidopsis thaliana* (Ara) and *Dictyostellium discoideum* (Dic). Sources for sequences are (GenBank accession nos.) P09874 (human), P18493 (bovine), P27008 (rat), P11103 (mouse), P26446 (chicken), P31669 (*X. laevis*), P35875 (*D. melanogaster*), D16482 (*S. peregrina*), Z47075 (*C. elegans*), Z48243 (*A. thaliana*); the sequence for *D. discoideum* is from [41]. (B) The first line represents the human PARP C-terminal extremity (residues 1000–1014). Mutants are named on the left of the panel and their activity is shown on the right (+, wild-type activity; –, no activity).

Interestingly, this residue was previously identified as a critical amino acid for catalytic activity [12,21].

Peptide 3: FYTLIPHPFGMKPPLL (residues 736–752)

This peptide corresponds to residues Phe⁷³⁶–Leu⁷⁵² (Figure 4). For this peptide, radiolabel was detected in a low yield at positions Thr⁷³⁸, Ile⁷⁴⁰, His⁷⁴², Phe⁷⁴⁴, Met⁷⁴⁶ and Pro⁷⁴⁹, with a maximum reached for Met⁷⁴⁶. The explanation of these results remains unclear. This peptide seems to be non-specifically labelled.

Site-directed mutagenesis, expression, partial purification and kinetic parameters of PARP proteins mutated in the photolabelled residues

To evaluate the role of the photolabelled residues in the catalytic process, point mutations were introduced into the pTG161 PARP expression vector encoding the full-length enzyme [21]. Crude lysates of TGE 900 cells expressing wild-type and mutated PARP proteins were generated and the amount of recombinant PARP was quantified immunologically (see the Experimental section). Table 2 displays the kinetic analysis of wild-type and mutated PARP proteins. The results clearly indicate that Trp¹⁰¹⁴ is not a crucial residue in terms of catalytic activity, despite being a strong site of photolabelling. The importance of the extreme C-terminal region of PARP was further investigated by systematic alanine scanning and deletion mutagenesis in the region 1007–1014. The results, summarized in Figure 5(B), indicate that none of these residues alone is crucial for catalytic activity, but the deletion of all of the sequence 1007–1014 abolishes catalytic activity.

In contrast with the results observed with Trp¹⁰¹⁴, both conservative (K893R) and non-conservative (K893I) mutations of Lys⁸⁹³ strongly affect the k_{cat} of the recombinant PARP as previously reported [12], thus confirming the importance of this residue for catalysis.

DISCUSSION

Since the original work of Shizuta and co-workers [30], it has been known that the 54 kDa C-terminal domain of the calf thymus PARP contains the site for binding the substrate, NAD⁺. In the present study we have used the photoaffinity probe 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ to specifically label amino acid residues in the catalytic domain of human PARP. The validity of this approach has been established with enzymes such as glutamate and lactate dehydrogenases that use NAD⁺ as a cofactor [28,31] and with mono(ADP-ribosyl)ating toxins using NAD⁺ as a cleavable substrate [32–34]. In the latter case, the NAD⁺-binding site of diphtheria toxin (DT) fragment A was photolabelled with unsubstituted NAD⁺ and the Glu¹⁴⁸ residue was found to be contiguous with the nicotinamide moiety of bound NAD⁺ [32]. This result has been confirmed by the recent determination of the crystallographic structure of the DT–NAD⁺ complex, showing that the γ -methylene carbon atom of Glu¹⁴⁸ and C-6 of the nicotinamide ring of NAD⁺ are 4.1 Å (0.41 nm) apart in the complex [35].

We have shown that photoinsertion of 2-azido-NAD⁺ in the human PARP catalytic domain is dependent on UV irradiation and leads to complete inactivation of the enzymic activity after 30 s of irradiation. Saturation experiments to determine stoichiometry indicate that approx. 0.73 mol of photoprobe is bound per mol of catalytic domain, suggesting that PARP contains a single NAD⁺-binding site. Among the different competitors used to test the specificity of the photoinsertion reaction, 3-aminobenzamide was found to be the strongest, thus confirming that the photoprobe was indeed targeted to the active site. However, our experiments cannot completely rule out the possibility of photolabelling of other sites in the protein by linkages not stable to sample preparation.

Microsequencing of the two major peptides photolabelled with 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ identified two potential sites for adenine binding: Trp¹⁰¹⁴, the C-terminal residue, and Lys⁸⁹³, which was

previously shown to be critical for activity [12]. As the binding stoichiometry of the photoprobe to the catalytic domain is slightly less than 1:1, the identification of two putative binding sites for 2-azido- $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ is difficult to reconcile with a unique NAD⁺ site, unless both Trp¹⁰¹⁴ and Lys⁸⁹³ can occur in close proximity to NAD⁺. This hypothesis should be compatible with a minimal distance between the two photolabelled residues. The crystallographic structure of the chicken PARP catalytic domain has been recently solved at 2.4 Å (0.24 nm) resolution in the presence of the nicotinamide analogue PD128763 [15]. Interestingly, the NAD⁺-binding site, which contains the fold β - α -loop- β - α , is mostly included in a conserved block of 50 residues (859–908) forming the PARP signature (Figure 6). In spite of a weak sequence similarity [36,37], this NAD⁺ fold is in fact superimposable on that of several toxins, including DT [35,38,39] and *Pseudomonas aeruginosa* exotoxin A (ETA) [40]. Therefore a unique and novel NAD⁺ fold is used both by mono(ADP)- and poly(ADP-ribosyl)ating enzymes. Given the similarity of their active sites and of the position of the nicotinamide moiety in DT, ETA and PARP, a model of NAD⁺ based on the observed nicotinamide analogue PD128763 as bound to PARP and on AMP as bound to ETA has been superimposed on the PARP active site (residues 859–908) (Figure 6). In this model the C α of Lys⁸⁹³ is located at a distance of only 10 Å from the nearest atom of the adenine ring (N-3), confirming that Lys⁸⁹³ is a likely candidate for NAD⁺ binding.

It was not possible to determine the position of the second site of photolabelling, Trp¹⁰¹⁴, with respect to the PARP active site because the last four residues (1011–1014) were not visible in the crystallographic structure [15]. Considering the fact that an extensive mutational analysis of the region 1007–1014 did not affect the catalytic activity unless the entire group of residues was deleted (Figure 5B), one can speculate that Trp¹⁰¹⁴ present at the extreme C-terminal part of PARP could be in contact with the adenine ring as an alternative site, perhaps more accessible than Lys⁸⁹³. In terms of sequence conservation during evolution, the alignment of 11 PARP sequences (residues 1000–1014) displayed in Figure 5(A) clearly shows that the region KTSWL is not conserved. In contrast, the strong conservation of Lys⁸⁹³ from human to *Arabidopsis thaliana* [42] within the PARP signature and also in the DT active site argues for a crucial role of this residue in catalysis [12]. Co-crystallization experiments of

Figure 6 Plot of the C α of residues 859–908 of PARP showing the side chain of Lys⁸⁹³

Stereo view of a model of bound NAD⁺ together with part of the C α backbone of PARP and the side chain of Lys⁸⁹³. The backbone represents the PARP 'signature sequence' (residues 859–908). The model of NAD⁺ is based on the observed nicotinamide analogue inhibitor PD128763 as bound to PARP [15] superimposed on PARP. The distance between Lys⁸⁹³-C α and the C-2 atom of adenine is 10 Å (1 nm).

complexes between the PARP catalytic domain and NAD⁺ are now in progress to locate, at the atomic level, residues contacting the substrate; in this respect the determination of photolabelled residues with the analogue 2-azido-NAD⁺ is helpful and complementary to the crystallographic approach.

We thank Transgène (Strasbourg) for the gift of the *E. coli* strain TGE900 and for the pTG161 expression vector. This work was supported in part by NIH Grant CA43894 to M.K.J. and by the Ligue Nationale Contre le Cancer and by l'Association pour la Recherche Contre le Cancer to G.d.M. and J. M.-d.M.

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