Evidence for the participation of histidine residues located in the 56 kDa C-terminal polypeptide domain of ADP-ribosyl transferase in its catalytic activity

Pal. I. Bauer[1], Kalman G. Buki[1] and Ernest Kun[2]

[1] *Laboratory for Environmental Toxicology and Chemistry, Romberg Tiburon Center, San Francisco State University, Tiburon, CA 94920, USA* and [2] *Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132, USA*

Received 20 August 1990

Purified ADPRT protein was inactivated by the histidine specific reagent diethylpyrocarbonate, binding to two histidine residues, or by a relatively histidine selective photoinactivation method. Inactivation with up to 1.3 mM diethylpyrocarbonate was reversible by hydroxylamine. Enzymatic inactivation coincided with the loss of binding capacity of the enzyme protein to benzamide affinity matrix but not to DNA cellulose. Labelled diethylpyrocarbonate was identified exclusively in the 56 kDa carboxyl-terminal polypeptide where 2 out of 13 histidine residues were modified by this reagent. It is proposed that histidine residues in the 56 kDa polypeptide may participate as initiator sites for polyADP-ribosylation.

ADP-ribosyltransferase; Diethylpyrocarbonate; Histidine residue; ADP-ribosylation; Photoactivation; Catalytic domain of ADPRT

1. INTRODUCTION

Extensive studies, primarily from the laboratory of Hayaishi (reviewed in [1,2]) partially characterized the mechanism of protein ADP-ribosylation reactions catalyzed by ADP-ribosyl transferase (ADPRT; EC 2.4.2.30) by the identification of a glutamate carboxyl ester bond [3] responsible for the covalent binding of poly(ADP-ribose) to histone H1, and it was reasonably assumed that this carboxyl ester is the chemical structure of any protein-(ADPR)n adduct. This ester bond exhibits stability to pH 7–8 since (ADPR)n protein adducts can be quantitatively isolated at this pH [4,5], but is unstable to alkaline hydroxylaminolysis, thus behaves like a moderately stable ester. Some unsolved problems remained, especially the ADP-ribosylation of proteins other than ADPRT appeared obscure, since one has to assume the transfer of an ADP-ribose moiety from an ADPRT-ADPR adduct to other protein acceptors, because it has been demonstrated that once an oligomer chain is formed it can no longer be translocated from ADPRT to secondary acceptors [6]. In pursuit of this question we have identified a highly base unstable mono ADPR-ADPRT adduct that is formed at nanomolar concentrations of NAD [7] by its isolation at pH 6.0. This mono ADP-ribose-ADPRT adduct rapidly decays at pH 7.0 and above and is only detectable at nM NAD concentrations and exhibits propensities that could be consistent with a postulated initiation adduct that can be *trans*-ADP-ribosylated to the more stable glutamate carboxyl ester product [7], either on the ADPRT molecule itself or on another protein that binds to ADPRT. We report here that reagents (diethylpyrocarbonate, abbr.DEP) and procedures (light inactivation) that are known to exhibit a considerable degree of selectivity towards histidine, inactivate ADPRT and inhibit the binding of ADPRT to benzamide affinity columns but not to DNA-affinity matrices. We also show with the aid of labelled DEP that covalent binding to histidine residues takes place exclusively in the C-terminal polypeptide domain of ADPRT [8], which is distinct from the blocked amino terminal 29 kDa domain of the protein known to contain Zn^{2+} finger polypeptides [9–11]. From the coincidence of this molecular topography of DEP binding with the selective inhibition of binding to the benzamide-Sepharose affinity column [8], that is specific for the nicotinamide binding of NAD, we conclude that two histidine residues in the 56 kDa polypeptide domain are probable initiator sites of the base unstable ADP-ribose-ADPRT initiator adduct formation.

Correspondence address: E. Kun, Romberg Tiburon Center, POB 855, 3150 Paradise Drive, Tiburon,CA 94920, USA

2. EXPERIMENTAL

The selective modification of histidinyl residues by diethylpyrocarbonate was carried out by standard procedures [12,13]. Photoinactivation of histidinyl residues was performed by the Rose-Bengal technique [12,14,15]. The ADPRT protein was isolated by a modification of a published method [16], the modification described as follows. The 35–65% ammonium sulfate cut was directly loaded onto an Affigel 10 matrix without prior removal of DNA by the Reactive Red and hydroxylapatite steps. This modification increased the yield of ADPRT by two-fold and significantly reduced the time of

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

operation. The Affigel 10 matrix was prepared by reacting 800 mg of *m*-aminobenzamide with 25 ml Affigel 10 resin (BioRad Co.) overnight, followed by extensive washing with 1 M KH_2PO_4 and K_2HPO_4, finally with buffer B [16]. The adsorbed enzyme was eluted with 1 mM methoxybenzamide, and coenzymic DNA subsequently removed by hydroxylapatite [16] equilibrated with buffer A (10 ml column volume, 200 ml buffer A). Methods of ADPRT assay and the isolation of polypeptide fragments of ADPRT were the same as reported [8,16]. ^{14}C-labelled DEP (9.4 mCi/mmol) was purchased from Sigma and $^{65}ZnCl_2$ from DuPont. The exchange of $^{65}Zn^{2+}$ in ADPRT was assayed as follows. The $^{65}Zn^{2+}$ (0.2 mM, 536 mCi/mmol) was incorporated into ADPRT by overnight incubation in 50 mM Tris-HCl, pH 8.1, 100 mM KCl at 6°C. The ADPRT concentration of the incubate was 0.33 mg protein/ml. The buffer medium and unbound $^{65}Zn^{2+}$ was then exchanged to 20 mM Tris-HCl, pH 8.1, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 mM KCl in a Centricon centrifugal filtration device. Incorporation of $^{65}Zn^{2+}$ into ADPRT was determined by a nitrocellulose filter (Schleicher & Schuell; 0.45 μm pore size) that retained ADPRT, and $^{65}Zn^{2+}$ on the filter was assayed by radiochemistry.

3. RESULTS

The rates of inactivation of ADPRT by increasing concentrations (from 0.06 to 2.5 mM) of DEP are illustrated in Fig. 1A. Fig. 1B shows a logarithmic relationship between $t_{1/2}$ of inactivation and the concentration of DEP. Neutral hydroxylamine (pH 7.4) reactivated ADPRT that was inactivated by pretreatment with DEP up to 1.3 mM for 10 min (Fig. 1C).

Photoinactivation of ADPRT in the Rose-Bengal system exhibited a dye concentration and time of irradiation dependence (Fig. 2).

ADPRT was labelled with [^{14}C]DEP (see legend of Fig. 3) and component polypeptides of ADPRT obtained by plasmin digestion were isolated by gel electrophoresis [8]. Fig. 3 shows the Coomassie stained polypeptides (A) and the autoradiography of labelled polypeptides (B). The unhydrolyzed purified ADPRT protein contained traces of labelled polypeptide degradation impurities, but plasmin digestion and electrophoresis clearly identified two main polypeptides, 56 kDa and 42 kDa, as the polypeptides containing covalently bound DEP residues (Fig. 3B). We have shown earlier that the 42 kDa polypeptide is the proteolytic product of the 56 kDa carboxy-terminal peptide [8], thus results given in Fig. 3 indicate that labelled histidine residues are present in the carboxy-terminal polypeptide domain of ADPRT. The histidine containing Zn^{2+} finger polypeptides reside in the 29 kDa basic blocked terminal polypeptide [9–11], yet no labelled DEP residue was detectable in this polypeptide domain. In order to ascertain the unreactivity of the histidine present in the Zn^{2+} finger polypeptide towards DEP, we assayed the stability of incorporated Zn^{2+} in ADPRT following DEP inactivation. The $^{65}Zn^{2+}$ con-

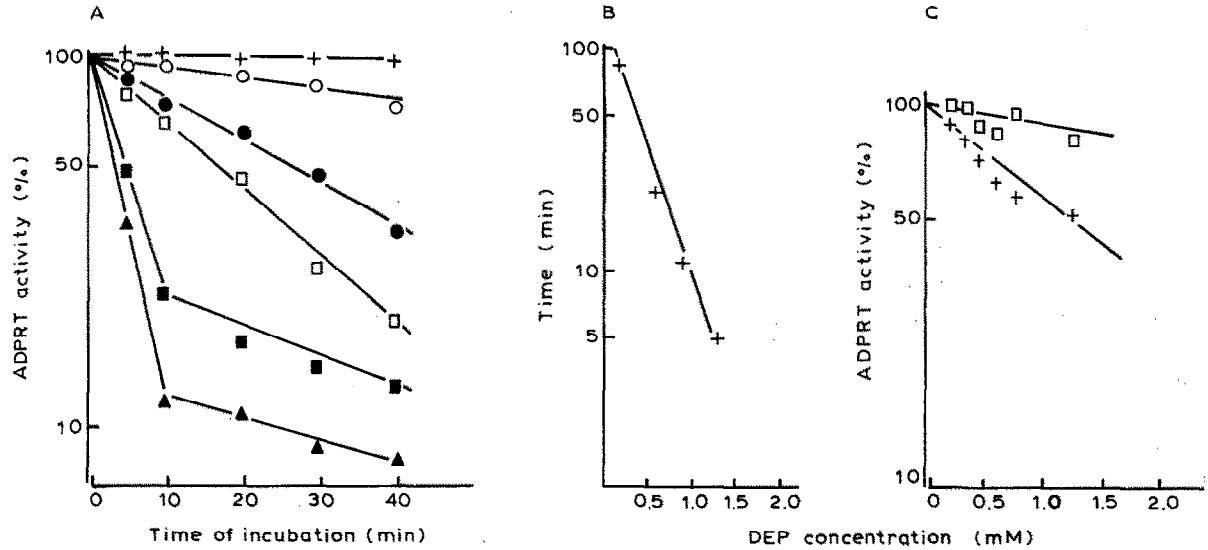

Fig. 1. Inactivation of ADPRT by diethylpyrocarbonate (DEP) and its reversal by hydroxylamine. (A) Inactivation. ADPRT (18 μg in 23 μl of 0.1 M phosphate buffer, pH 6.0) was incubated at 4°C for various times with an ethanolic solution of DEP at varying concentrations (0–2.5 mM DEP). At given times 4 μl aliquots were withdrawn, quenched with 4 μl of 12 mM imidazole and assayed for polymerase enzymatic activity (see Fig. 1C, [7,8]). (+—+) 0.06 mM DEP; (○—○) 0.31 mM DEP; (●—●) 0.62 mM DEP; (□—□) 0.82 mM DEP; (■—■) 1.25 mM DEP; (▲—▲) 2.5 mM DEP. (B) Correlation between the time required to obtain 50% enzyme inhibition and the concentration of DEP, plotted on a semilogarithmic scale. Results were calculated from (A). (C) Reversal by hydroxylamine. First ADPRT was inactivated by varying final concentrations of DEP (up to 1.3 mM) by adding 1 μl of ethanolic DEP solution to each 24 μl aliquot of ADPRT solution, consisting of 20 μg ADPRT/24 μl in 0.1 M phosphate buffer, pH 6.0, and incubation for 10 min at 4°C. At this time 2 μl of imidazole were added to quench DEP and incubation continued for another 10 min. Then two 10 μl aliquots were withdrawn from each test system and treated with either 10 μl phosphate buffer or 10 μl of 500 mM hydroxylamine, adjusted to pH 7.4, for 22 h at 4°C. 2 μl aliquots were withdrawn and the assay volume increased to 200 μl with assay buffer in order to avoid the ADPRT inhibitory effects of hydroxylamine and imidazole, which was found to be only 10% under given conditions. In Fig. 1C the remaining ADPRT activities of DEP-treated ADPRT samples are shown, (□—□) with, or (+—+) without hydroxylamine treatment, as a function of varied DEP concentrations. The ADPRT activities determined in the controls (taken as 100%) were determined after incubation with ethanol only instead of ethanolic DEP and further treatment as described above.

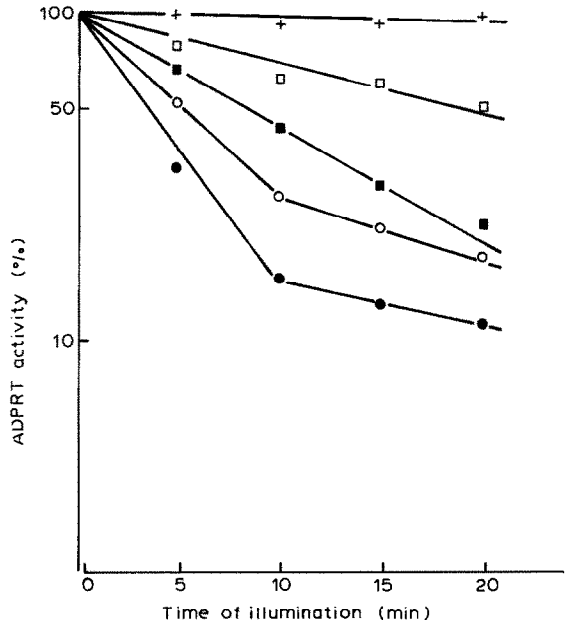

Fig. 2. Photoinactivation of ADPRT in the presence of Rose-Bengal. To an ADPRT solution containing 25 μg of protein in 50 μl of Tris-HCl buffer (pH 8.0), 6 μl of aqueous Rose-Bengal dye solution was added. The final concentration of the dye varied between 0–160 μg/ml. The samples were placed onto ice and illuminated from a 20 cm distance with a 500 W spotlight. After different times of irradiation 2 μl samples were withdrawn to determine remaining ADPRT activities. Controls were either a system which was illuminated without the dye, or a solution of ADPRT containing 150 μg/ml of Rose-Bengal but kept in dark throughout. (+—+) control (no dye or no irradiation); (□—□) 40 μg/ml Rose-Bengal; (■—■) 80 μg/ml Rose-Bengal; (○—○) 120 μg/ml Rose-Bengal; (●—●) 160 μg/ml Rose-Bengal.

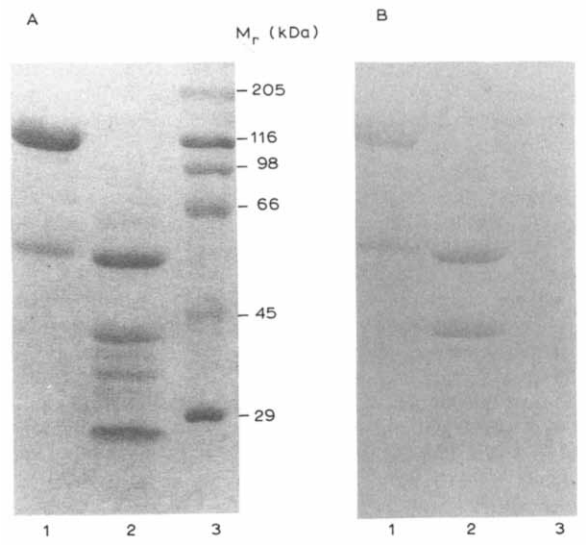

Fig. 3. The labelling of ADPRT with [^{14}C]DEP and isolation of polypeptides containing labelled DEP. To 100 μl of a solution of ADPRT (80 μg protein in 0.1 M phosphate buffer, pH 6.0), [^{14}C]DEP (final concentration 0.68 mM, 10 μCi dissolved in ethanol) was added and the mixture incubated for 20 min at 4°C. At the end of the incubation 10 μl of 120 mM imidazole was added and incubation continued for 10 min at 4°C. The sample was filter-dialysed through an Amicon cartridge, the buffer changed to 50 mM Hepes buffer, pH 7.4, containing 100 mM NaCl and 7 mM 2-mercaptoethanol. 20 μg of the DEP modified protein was digested with plasmin (1 μg) for 20 min as described earlier [8]. At the end of the incubation an equal volume of sample buffer (Laemmli) was added, and the sample loaded onto a 10% acrylamide SDS-PAGE gel. After electrophoresis the gel was fixed with 20% trichloroacetic acid, stained with Coomassie brilliant blue, destained in acetic acid, dried, and autoradiographed for one week at −40°C. (A) Coomassie-stained profile of the electropherogram. Lane 1 = DEP-modified ADPRT; lane 2 = plasmin digested DEP-modified ADPRT; lane 3: molecular weight markers. (B) Autoradiogram of the same gel.

tent of native ADPRT was 2240 ± 120 dpm and that of the DEP treated enzyme 2480 ± 130 dpm per μg ADPRT (duplicates). These results confirm the apparent selectivity of histidine labelling by DEP in the 56 kDa polypeptide.

We determined the biochemical consequences of histidine modification by DEP on the DNA and benzamide binding ability of ADPRT [8]. The technique employed is described in the legend of Fig. 4 which shows the gel electrophoretic identification of ADPRT under varying conditions. It is apparent that covalent modification of histidinyl residues in the 56 kDa polypeptide domain inhibited the binding of ADPRT to the benzamide column but not to the DNA-affinity matrix. The small amount of ADPRT eluted by methoxybenzamide (lane 12) represents the DEP unreacted portion of the protein (see Fig. 1A).

From the labelling and spectral analyses of ADPRT it was calculated that 2 mol of histidine were covalently modified per mol of ADPRT, coinciding with enzyme inactivation and inhibition of binding to the benzamide matrix.

4. DISCUSSION

The coincidence of selective and hydroxylamine reversible inactivation of ADPRT by DEP, and the simultaneous imidazole specific photoinactivation provide chemical evidence for the participation of histidine residues in the ADPRT catalyzed polyADP-ribosylation reaction. It is known from the cDNA sequence [17] that out of 19 histidine residues per molecule of ADPRT 5 reside in the 29 kDa amino terminal polypeptide that contains 2 Zn fingers [9–11], one in the 36 kDa polypeptide [8] and 13 in the carboxy terminal 56 kDa polypeptide. It is of interest that DEP reacts with only two histidine residues of the 56 kDa polypeptide but not with imidazole molecules present in the 29 kDa polypeptide domain. This apparent selectivity is unexplained, unless one considers macromolecular structural factors such as dimerization [18] that may determine this selectivity towards DEP under given experimental conditions. Our results thus provide

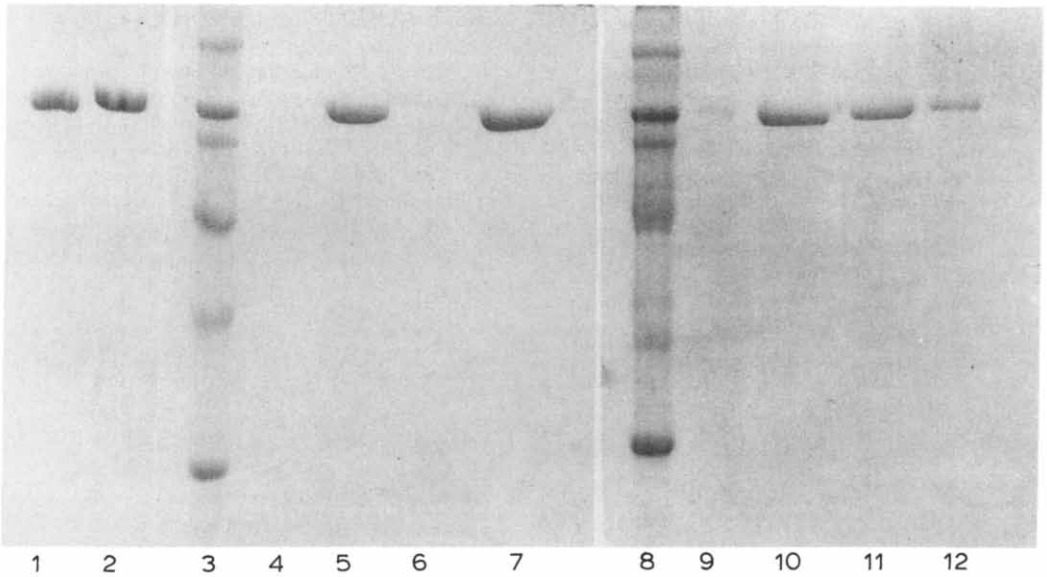

Fig. 4. The effect of modification of ADPRT by DEP on its binding to DNA-cellulose and benzamide-Sepharose affinity columns. Solutions of ADPRT (50 μg ADPRT in 100 μl 50 mM phosphate buffer, pH 6.0) were treated with either 1 μl ethanol (native ADPRT) or 1 μl of ethanolic solution of 66 mM DEP (DEP-modified ADPRT) for 40 min at 4°C. Then 20 μl of 120 mM imidazole was added to quench excess DEP, and incubation was continued for 15 min at 4°C. The samples were then diluted with 1 ml of chromatographic buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM 2-mercaptoethanol) and 0.47 ml aliquots were loaded onto either DNA-cellulose or benzamide-Sepharose columns (0.5 ml bed volume each) that were equilibrated with the same buffer [8]. The rest was saved for gel electrophoresis (lanes 1 and 2). The columns were washed with 2 × 2 ml of buffer and the flow-through and washing eluates combined (lanes 4, 9, 6 and 11 in the electropherogram). The matrix-bound proteins were eluted in the case of DNA cellulose with 1 M NaCl dissolved in the buffer, or with 1 mM of methoxybenzamide in the case of the benzamide-Sepharose matrices (lanes 5, 10, 7 and 12). The volume of the eluents was 4 ml. The collected fractions were concentrated in Centricon C30 cartridges, volumes adjusted to 100 μl, and aliquots (25 μl) mixed with the sample buffer of the Laemmli SDS-PAGE system and loaded onto a 10% acrylamide SDS-PAGE gel (cf. [16]). After electrophoresis the gels were stained with Coomassie blue and destained in 10% acetic acid. Lane 1 = native ADPRT; lane 2 = DEP modified ADPRT; lane 3 = molecular weight markers (myosine, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase from top to bottom); lane 4 = native ADPRT, DNA-column flow-through and wash; lane 5 = native ADPRT, DNA-column 1 M NaCl eluate; lane 6 = DEP-modified ADPRT, flow-through and wash of DNA column; lane 7 = DEP-modified ADPRT, 1 M NaCl eluate of DNA column; Lane 8 = molecular weight markers; Lane 9 = native ADPRT, flow-through and wash of benzamide-Sepharose column; lane 10 = native ADPRT, 1 mM methoxybenzamide eluate of benzamide-Sepharose column; lane 11 = DEP modified ADPRT, flow-through and wash of benzamide-Sepharose column; lane 12 = DEP modified ADPRT, 1 mM methoxybenzamide eluate of benzamide-Sepharose column.

preliminary evidence for the localization of the catalytic site of ADPRT in the 56 kDa carboxy-terminal polypeptide domain, coinciding with a previous suggestion [19], which is based on specific thiol labelling of ADPT. The participation of both SH and histidyl residues in the formation of an unstable ADPR-histidyl adduct appears reasonable and provides the first indication of the chemical nature of the previously observed highly base unstable mono ADPR adduct [7]. However, exact localization and definition of catalytic function of the two histidinyl residues in the 56 kDa polypeptide probably involved in the first step of the polyADP-ribose polymerase reaction require more detailed studies involving site-oriented mutations in ADPRT at various histidine residue sites, and our results only point to the amino acid residues where these genetic experiments may prove successful.

Acknowledgements: This research was supported by Grant AFOSR-89-0231 received from the Air Force Office of Scientific Research. We thank Dr E. Kirsten for help in preparing the manuscript.

REFERENCES

[1] Hayaishi, O. and Ueda, K. (1977) Annu. Rev. Biochem. 46, 95–116.
[2] Althaus, F.R. and Richter, C. (1987) ADP-Ribosylation of Proteins Mol. Biol. Biochem. and Biophys. Ser. 37, (Springer, Heidelberg).
[3] Ogata, N., Ueda, K., Kagamiyama, H. and Hayaishi, O. (1980) J. Biol. Chem. 255, 7616–7620.
[4] Okayama, H., Ueda, K. and Hayaishi, O. (1978) Proc. Natl. Acad. Sci. USA 75, 1111–1115.
[5] Romaschin, A.D., Kirsten, E., Jackowski, G. and Kun, E. (1981) J. Biol. Chem. 256, 7800–7805.
[6] Kawaichi, M., Ueda, K. and Hayaishi, O. (1981) J. Biol. Chem. 256, 9483–9489.
[7] Bauer, P.I., Hakam, A. and Kun, E. (1986) FEBS Lett. 195, 331–338.
[8] Buki, K.G. and Kun, E. (1988) Biochemistry 27, 5990–5995.
[9] Mazen, A., Menissier-deMurcia, J., Molinete, M., Simonin, F., Gradwohl, G., Poirier, G. and deMurcia, G. (1989) Nucleic Acids Res. 17, 4689–4698.
[10] Menissier-deMurcia, J., Molinete, M., Gradwohl, G., Simonin, F. and deMurcia, G. (1989) J. Mol. Biol. 210, 229–233.
[11] Gradwohl, G., Menissier-deMurcia, J., Molinete, M., Simonin, F., Koken, M., Hoeijmakers, J.H.J. and deMurcia, G. (1990) Proc. Natl. Acad. Sci. USA 87, 2990–2994.

[12] Lundblad, R.L. and Noyes, C.M. (1984) Chemical Reagents for Protein Modification Vol. 1, p. 108.
[13] Miles, E.W. (1977) Modification of Histidinyl Residues in Proteins by Diethylpyrocarbonate in Methods Enzymol. 47, 431–442.
[14] Fahnestock, S.R. (1975) Biochemistry 14, 5321–5329.
[15] Auron, P.E., Erdelsky, K.J. and Fahnestock, S.R. (1987) J. Biol. Chem. 253, 6893–6900.
[16] Buki, K.G., Kirsten, E. and Kun, E. (1987) Anal. Biochem. 167, 160–166.
[17] Ushida, K., Morita, T., Sato, T., Yamashita, R., Nogushi, S., Suzuki, H., Nyunoya, H., Miwa, M. and Sugimura, T. (1987) Biochem. Biophys. Res. Commun. 148, 617–622.
[18] Bauer, P.I., Buki, K.G., Hakam, A. and Kun, E. (1990) Biochem. J. 270, 17–26.
[19] Kameshita, I., Matsuda, Z., Taniguchi, I. and Shizuta, Y. (1984) J. Biol. Chem. 259, 4770–4776.