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ADP-Ribosylation of ADPR-Transferase and Topoisomerase I in Intact Mouse Epidermal Cells JB6†

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ABSTRACT: Poly(ADP-ribosylation) [poly(ADPR)] is a posttranslational modification of chromosomal proteins that affects the structural and functional properties of chromatin. We have studied poly(ADPR) of ADPR-transferase and topoisomerase I in intact mouse epidermal cells JB6 (clone 41) by a combination of affinity chromatography on phenylboronate and immunoblotting with monoclonal antibodies against poly(ADPR) chains and polyclonal antibodies against ADPR-transferase and topoisomerase I, respectively. Constitutive, steady-state poly(ADPR) substitution of ADPR-transferase was estimated at 4% and that of topoisomerase I at 0.1%. Active oxygen produced extracellularly by xanthine–xanthine oxidase and the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine transiently increased the level of poly(ADPR) substitution of these enzymes by a factor of 6–10. While the poly(ADPR) substitution of ADPR-transferase remained elevated after 60 min of incubation, the poly(ADPR) substitution of topoisomerase I had returned to control values within this time. Benzamide (100 μM) partially prevented the stimulation of poly(ADPR) synthesis by these agents. We speculate that self-inactivation of ADPR-transferase by poly(ADPR) represents a feedback mechanism that has the function to avoid excessive poly(ADPR) synthesis and concomitant NAD and ATP depletion. Inactivation of topoisomerase I in the neighborhood of DNA breakage may temporarily shut down DNA replication and allow DNA repair to occur.

Constitutive poly(ADP-ribosylation) appears to play a role in several facets of chromatin and DNA metabolism (Althaus & Richter, 1987; Althaus et al., 1985). The relatively rapid turnover of poly(ADPR) chains renders this posttranslational protein modification particularly suitable for regulatory purposes. Poly(ADPR) can have both structural and functional effects. For example, the poly(ADPR) of histones may alter nucleosomal conformation (Niedergang et al., 1985; Poirier et al., 1982; Adamietz & Rudolph, 1984) and higher order chromatin structure in relationship to replication, repair, recombination, and transcription (Althaus & Richter, 1987; Althaus et al., 1985). Support for a structural role of poly(ADPR) derives from the observation that a majority of the poly(ADP-ribosylated) proteins are associated with the nuclear matrix (Adolph & Song, 1985a,b; Wesierska-Gadek & Sauerman, 1985; Cardenas-Corona et al., 1987). Poly(ADPR) of enzymes involved in DNA metabolism such as topoisomerases (Ferro & Olivera, 1982, 1984; Ferro et al., 1983), ligases (Ohashi et al., 1983; Teraoka et al., 1986), DNA-dependent RNA polymerase (Muller & Zahn, 1976), transcription factors, and ADPR-transferase itself (Adamietz & Rudolph, 1984; Kawaichi et al., 1981; Jump & Smulson, 1980; Singh et al., 1985) may regulate their activities. Poly(ADPR) is unique because it is stimulated as a consequence of DNA strand breakage caused by a variety of mutagens and carcinogens. Therefore, besides its physiological functions, poly(ADPR) is bound to participate in the processsing and expression of DNA damage.

Active oxygen (AO) and other oxidants act as tumor promoters (Cerutti, 1985, 1986). The mechanism of action of oxidant promoters appears to involve the modulation of the expression of growth- and differentiation-related genes and cytotoxic effects (Muehlematter et al., 1988; Crawford & Cerutti, 1988). AO and poly(ADPR) affect the redox state and energy metabolism of the cell, and AO induces DNA strand breaks that activate ADPR-transferase. Therefore, AO-induced poly(ADPR) of chromosomal proteins is likely to play a role in oxidant promotion (Singh et al., 1985).

In the present work we have focused on poly(ADPR) of ADPR-transferase, the biosynthetic enzyme that polymerizes NAD residues, and topoisomerase I in intact mouse epidermal cells JB6 (clone 41). Our results give insights into the steady-state levels of constitutive poly(ADPR) of these enzymes and of poly(ADPR) in response to AO and the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) that was used as a "positive" control (Cerutti et al., 1987).

Materials and Methods

Materials

Xanthine and benzamide were purchased from Sigma, and xanthine oxidase was purchased from Böhringer Mannheim. Phenylboronate agarose matrix gel PBA30 and ultrafiltration

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cells and filters, YM10, were obtained from Amicon. Nitrocellulose, 0.2 μm, was obtained from Cellpore, [^{3}H]leucine, ^{125}I-labeled donkey anti-rabbit Ig, and ^{125}I-labeled sheep anti-mouse Ig were purchased from Amersham. JB6 clone 41 mouse epidermal cells were received from Dr. N. Colburn (U.S. National Cancer Institute). Calf thymus topoisomerase I polyclonal rabbit antibodies were a gift from Dr. H. Vosberg (Max Planck Institute für Medizinische Forschung, Heidelberg, FRG). As shown in Figure 3 (lane 7) for control material that is not retained by the PBA30 phenylboronate column, this antibody only gives rise to a single band at 95 kDa. This result attests to its specificity and is in agreement with the work by Guldner et al. (1986). Calf thymus ADPR-transferase polyclonal rabbit antibodies were a gift from Dr. K. Ueda [Kyoto University, Japan; see Ikai and Ueda (1980)]. Monoclonal antibodies against poly(ADPR) were the generous gift of Dr. A. Belcredi (Osterreichisches Forschungszentrum Seibersdorf, Austria) who gave the following information regarding their preparation and properties [see Belcredi (1987)]. Poly(ADPR) antigen of variable chain length was synthesized in vitro by using isolated nuclei from mouse testis and thymus and injected into BALB/C mice. Monoclonal antibodies were prepared by standard procedures. The antibodies used in the present work were specific for poly(ADPR) and possessed minimal cross-reactivity with mono(ADP-ribose) and poly(adenosine).

Methods

Cell Culturing and Treatment Conditions. Monoclonal cultures of mouse epidermal cells JB6 (clone 41) were grown in 15-cm diameter Petri dishes in MEM (Gibco, Geneva) supplemented with 8% fetal calf serum and antibiotics in a 5% CO_2 atmosphere at 37 °C in a humidified incubator. The cells from several dishes were pooled and then replated at 3 × 10^5 cells/dish, [^{3}H]leucine (0.2 μCi/mL) was added, and the cultures were allowed to grow to 60% confluency. The MEM was replaced with medium 199, and a day later the cultures were exposed to an extracellular burst of active oxygen (AO) produced by the addition of 50 μg/mL xanthine plus 5 μg/mL xanthine oxidase. Control cultures received only 50 μg/mL xanthine. For comparison separate cultures were treated with the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at 5 μg/mL. In a series of experiments the ADPR-transferase inhibitor benzamide (100 μM) was added 15 min before the exposure to AO.

Preparation of Non-Histone Nuclear Proteins. After 20 min of treatment with MNNG and 30 or 60 min of exposure to AO, respectively, the cells were scraped with a rubber policeman and for each experimental condition the content of 30 dishes (approximately 1.5 × 10^8 cells) pooled for the preparation of nuclear proteins. Cell lysis was achieved by the incubation in a pH 3.1 buffer containing 25 mM sodium citrate/0.5 mM PMSF/0.5% Nonidet P-40/2 mM benzamide for 1 h at 0 °C essentially as described by Adamietz and Rudolph (1984). After low-speed centrifugation, the pellet was taken up in the same buffer and nuclei were released by 15–20 strokes in a Dounce homogenizer. The homogenate was diluted with an equal volume of 25 mM sodium citrate/0.2 mM PMSF/0.88 N sucrose, pH 3.1. The diluted homogenate was layered on top of an equal volume of the sucrose buffer, and the nuclei were pelleted by centrifugation in a swinging bucket rotor at 3200*g* for 7 min at 0 °C. Aliquots of the nuclear pellet were incubated for 60 min in 0.1 N NaOH/500 mM NaCl at room temperature, and the incubation was continued for 45 min at 30 °C. The samples were then neutralized with 1 N HCl and the acid-insoluble proteins precipitated with 0.25 M H_2SO_4 for 30 min at 4 °C. The remainders of the nuclear pellets were directly treated with 0.25 M H_2SO_4 without alkali pretreatment. The acid-insoluble material was taken up in 4 mL of a pH 6.5 buffer containing 6 M guanidinium chloride/50 mM sodium phosphate/5 mM mercaptoethanol and centrifuged for 16 h at 4 °C in a SW-60 rotor at 210000*g*.

Separation of Poly(ADP-ribosylated) Non-Histone Nuclear Proteins. The high-speed supernatant was brought to pH 8.2 by the addition of 200 μL of 1 M morpholine buffer, pH 8.6 (final morpholine concentration 50 mM). The poly(ADP-ribosylated) proteins were separated by adsorption to a column containing 8 mL of PBA 30 phenylboronate agarose matrix gel as described by Adamietz and Rudolph (1984). The column was washed with 20 mL of a pH 8.2 buffer composed of 6 M guanidinium chloride/50 mM morpholine. The material that remained adsorbed was removed from the column with 16 mL of 6 M guanidinium chloride/200 mM phosphate, pH 5.5. The entire procedure was carried out at 4 °C and was completed within 30 min. The pooled wash plus nonadsorbed material and the eluted adsorbed sample were concentrated and desalted by ultrafiltration using Amicon YM10 membranes. The concentrated material retained on the filters was dissolved with 200 μL of electrophoresis buffer, and aliquots were counted for their content in radioactivity.

Immunoblot Analysis of Poly(ADP-ribosylation) of Topoisomerase I and ADPR-Transferase. The proteins that had been retained by the PBA 30 affinity column and the proteins contained in the flow-through plus wash were analyzed by electrophoresis on 7% polyacrylamide gels with a buffer containing 4.5 M urea/0.2% SDS, pH 6.0, as described by Holtlund et al. (1983). The sizes of the applied samples were adjusted according to their radioactivity content. After the development of the gels, the proteins were electrotransferred overnight (0.3 mA, 15 V, 4 °C) to nitrocellulose filters in a pH 6.8 buffer containing 150 mM glycine/20 mM Tris base/0.2% SDS/15% methanol according to the method of Towbin et al. (1979). The filters were washed with distilled water. All subsequent steps were carried out in sealed plastic bags.

ADPR-Transferase. The nitrocellulose filter was saturated overnight at 4 °C on a rocker platform with a 10 mM Tris buffer, pH 7.4, containing 1× Denhardt's solution, 100 mM $MgCl_2$, 0.1% Triton X-100, and 0.5% BSA. Polyclonal rabbit anti-calf thymus ADPR-transferase antibodies at a dilution of 1:2000 were then added in the same buffer, and the incubation was continued for 12 h under the same conditions. The filters were then washed four times with TBS buffer before the addition of 1.5 × 10^5 cpm of ^{125}I-labeled donkey anti-rabbit Ig and incubation overnight at 4 °C. All immunoblots were developed by autofluorography using presensitized Amersham X-ray hyperfilm HP.

Topoisomerase I. The nitrocellulose filter was saturated as described above in an aqueous solution of 10% skimmed milk containing 0.1% NaN_3. Polyclonal rabbit anti-calf thymus topoisomerase I antibodies at a dilution of 1:750 in the same buffer were added, and the incubation was continued for 12 h at 4 °C. This antibody does not cross-react with ADPR-transferase (Dr. G. de Murcia, personal communication). After several washes with the same buffer, 2.5 × 10^5 cpm of ^{125}I-labeled donkey anti-rabbit Ig was added for incubation overnight at 4 °C.

Poly(ADPR). The nitrocellulose filter was saturated overnight in PBS containing 5% fetal calf serum/0.1% NaN_3 at 4 °C. A hybridoma supernatant containing monoclonal

antibodies against poly(ADPR) was then added at a 1:5 dilution in the same buffer, and the incubation was continued for 12 h at 4 °C before the filter was washed several times with PBS. Finally, the filter was reacted with ^{125}I-labeled sheep anti-mouse Ig (2.5×10^5 cpm) overnight at 4 °C.

Estimation of the Extent of Poly(ADP-ribosylation) of ADPR-Transferase and Topoisomerase I. As described above, total cellular proteins were labeled with [^{3}H]leucine, and according to our experimental design each culture dish contained equal amounts of radioactivity at the time of treatment. Equal aliquots of radioactivity of the non-histone nuclear protein preparations were applied to PBA-30 columns as described above, and on average 1.3–1.5% of the applied radioactivity remained adsorbed to the resin in controls and treated samples alike. Aliquots of the column flow-through and of the eluted adsorbed material were separated on polyacrylamide gels that were developed by the radioimmunoblot procedure described above. The intensities of the bands on the resulting autofluorograms were measured by densitometry. The amounts of [^{3}H]leucine radioactivity applied on the gels were directly proportional to the densitometer readings for radioimmunoblots with antibodies directed against ADPR-transferase and topoisomerase I, respectively. Therefore, the extent of poly(ADPR) substitution could be calculated from the amounts of [^{3}H]leucine radioactivity of the PBA-30 flow-through and adsorbed proteins applied on the gels and the resulting densitometer readings (see Tables I and II). The data reflect the extent of substitution of a particular protein independent of the number of substituents and of the lengths of the poly(ADPR) chains.

Results

We developed the following experimental design to study constitutive and induced poly(ADP-ribosylation) of ADPR-transferase and topoisomerase I in intact mouse epidermal cells JB6 (clone 41). As inducers of poly(ADPR) synthesis we used AO generated extracellularly by xanthine–xanthine oxidase (X/XO, 50 μg/mL X plus 5 μg/mL XO) and as a positive control the methylating agent MNNG (5 μg/mL). While kinetic studies were performed for AO, poly(ADPR) following MNNG was measured at its maximum, which is reached within 20 min under our experimental conditions (Muehlematter et al., 1988). To facilitate quantitation and sampling, monolayer cultures were labeled in their proteins with [^{3}H]-leucine. Acid-insoluble nuclear proteins were separated into poly(ADP-ribosylated) and non-poly(ADP-ribosylated) fractions by affinity chromatography on phenylboronate PBA-30 columns. These fractions were analyzed by the immunoblot technique with polyclonal antibodies against ADPR-transferase and topoisomerase I. Total poly(ADP-ribosylation) was monitored with a monoclonal antibody against poly(ADPR) chains. Throughout this study we included conditions with the ADPR-transferase inhibitor benzamide to gain insight into the turnover rates of constitutive poly(ADPR) chains and to ascertain that the material retained on the PBA-30 phenylboronate columns was enriched in poly(ADP-ribosylated) proteins. It should be noted that PBA-30 adsorbed material also contained unsubstituted proteins. This is evident from the fact that 1.3–1.5% of the total [^{3}H]leucine-labeled proteins were always retained on the column in untreated controls and AO- or MNNG-treated samples.

Figure 1A shows immunoblots of nuclear proteins with monoclonal antibodies against poly(ADPR) chains (lanes 1–6 contain equal amounts of [^{3}H]leucine radioactivities of PBA-30-purified protein, while lane 7 contains the PBA-30 flow-through of the untreated control). Only a single major band is visible in the 120-kDa region in the untreated control (lane 1) and probably consists of constitutively poly(ADP-ribosylated) ADPR-transferase (Adamietz & Rudolph, 1984; Kawaichi et al., 1981; Jump & Smulson, 1980; Singh et al., 1985). AO and MNNG treatment induces a strong accumulation of material in this band within 30 and 20 min, respectively. According to the densitometer readings shown on the left side of Figure 1A, the increase is estimated between 5- and 7-fold. The main band is flanked by higher and lower molecular weight bands. The smear above 120-kDa indicates the presence of high molecular weight material, the molecular weight being determined by the acceptor protein and the length of the poly(ADPR) chains. Additional distinct bands in the treated samples are found at 85 and 73 kDa and diffuse bands below 35 kDa. While the amount of poly(ADP-ribosylated) material in the 120-kDa region remains elevated 60 min after the AO treatment, the intensity of the other bands has been strongly reduced (lane 4). Benzamide (100 μM) strongly suppressed AO-induced poly(ADP-ribosylation) (lanes 3 and 5). The fact that the nonadsorbed material (lane 7) did not react with the antibody attests to the efficacy of the PBA-30 column in retaining poly(ADP-ribosylated) proteins. For further characterization the susceptibility of the poly(ADP-ribose) substituents to alkaline hydrolysis was tested. As shown in Figure 1B, incubation of the nuclear preparations with 0.1 N NaOH before acid precipitation and PBA-30 chromatography totally eliminated the bands on the immunoblots with anti-poly(ADPR) antibody.

Figure 2A gives the immunoblots of PBA-30 adsorbed and nonadsorbed nuclear proteins with polyclonal antibodies against ADPR-transferase. It is evident that AO caused a strong increase in the unique band in the 120-kDa region after 30 min of incubation (lane 2) that diminished in intensity after 60 min (lane 4). The addition to the cell cultures of benzamide (100 μM) strongly suppressed the reaction (lanes 3 and 5). As reported above for the immunoblots with anti-poly(ADPR) antibody, no protein bands are discernible when the nuclear preparations were preincubated with alkali (Figure 2B). Table I contains the levels of poly(ADPR) substitution independent of chain length. These values were calculated from the amounts of protein applied on each lane for PBA-30 adsorbed and nonadsorbed material (determined from the [^{3}H]leucine radioactivity) and the densitometry readings of the immunoblots. As reported under Methods, the densitometer readings were directly proportional to the amount of [^{3}H]leucine protein radioactivity applied on the gel. It follows that under steady-state conditions 4% of ADPR-transferase carries at least

Table I: Poly(ADP-ribosylation) of ADPR-Transferase by Active Oxygen (JB6 Clone 41)

	normalized densitometer readings[b]	poly(ADP-ribosylation) of ADPR-transferase (%)
adsorbed on boronate[a]		
control	12.2	4.0
X/XO, 30 min	115.9	37.9
X/XO + benzamide, 30 min	52.6	17.2
X/XO, 60 min	76.6	25.0
X/XO + benzamide, 60 min	46.6	15.2
MNNG, 20 min	40.5	13.2
nonadsorbed on boronate		
control	306.1	

[a] Cells exposed to active oxygen (X/XO 50/5 μg/mL), active oxygen plus benzamide (100 μM), or MNNG (5 μg/mL) for the indicated lengths of time. [b] Densitometer readings from Western blots normalized to 1% of the total nuclear proteins from 1.4×10^8 cells.

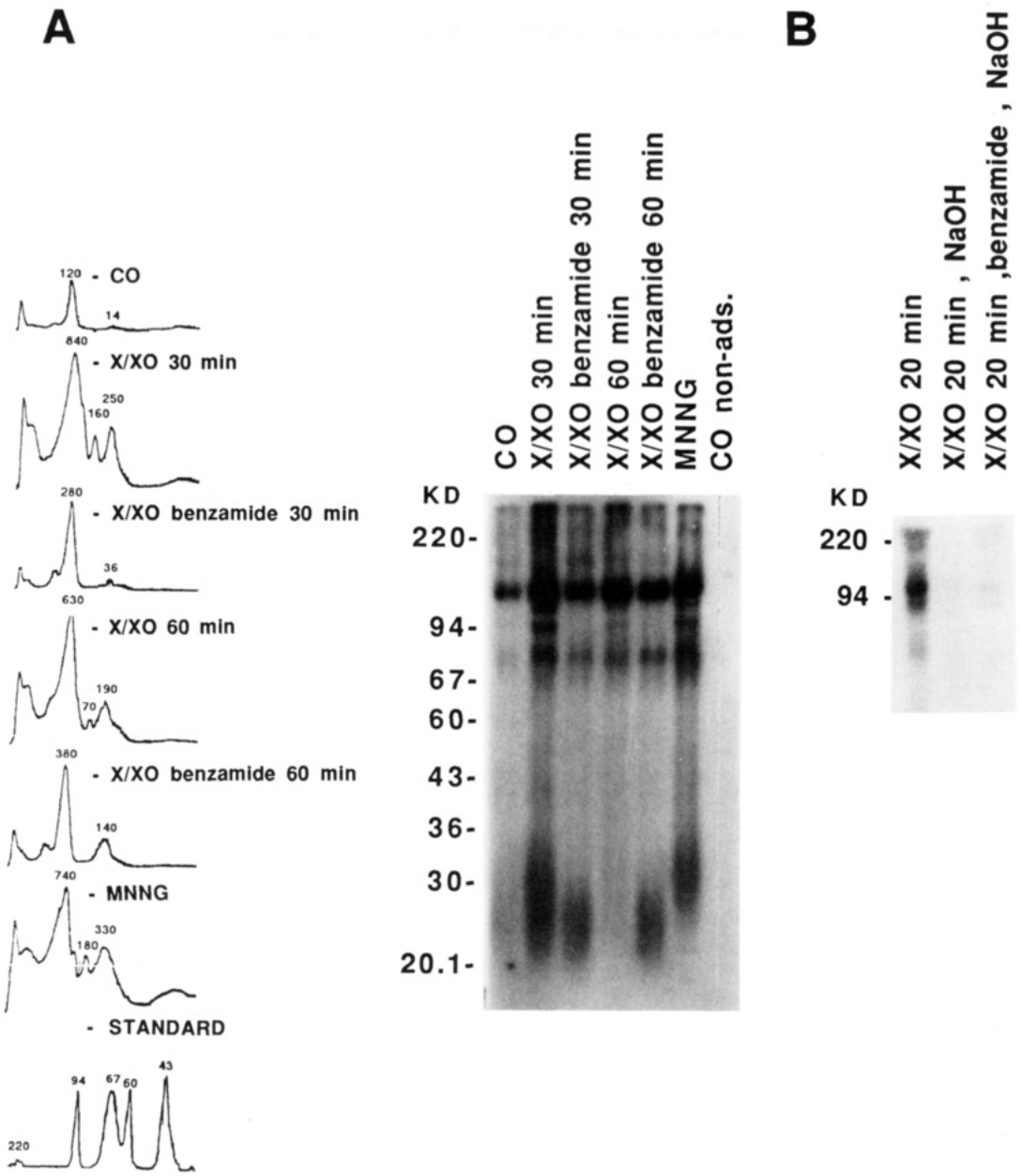

FIGURE 1: Poly(ADPR) immunoblots of acid-insoluble material from nuclear preparations of mouse epidermal cells JB6 (clone 41) that had been treated with active oxygen produced by xanthine–xanthine oxidase (50 μg/mL xanthine plus 5 μg/mL xanthine oxidase) for 30 min in the presence or absence of benzamide (100 μM) or with 5 μg/mL *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 20 min. The purification of the proteins by affinity chromatography and their separation on polyacrylamide gels was as described under Methods. (A) On the left side are shown autofluorograms of immunoblots with monoclonal mouse antibodies against poly(ADPR). The filters were reacted with ^{125}I-labeled sheep anti-mouse Ig. The right side gives the densitometer tracings of the immunoblots. The figures above the individual peaks denote the surface in arbitrary units. The densitometer tracing on the bottom derives from the peripheral part of the gel that contained molecular weight standards and was stained with Ponceau S stain. (B) Removal of poly(ADPR) chains from non-histone acceptor proteins by treatment of nuclear preparations with 0.1 N NaOH as described under Methods.

one poly(ADPR) chain, that a burst of AO generated by X/XO (50 μg/mL X plus 5 μg/mL XO) induced an approximately 10-fold increase in the fraction of substituted enzyme molecules in 30 min, and that this fraction later decreased. Benzamide (100 μM) suppressed the reaction by 55% at 30 min. MNNG (5 μg/mL, 20-min incubation) was much less efficient than AO in increasing the level of poly(ADPR) substitution of ADPR-transferase.

Figure 3 shows immunoblots with polyclonal antibodies against topoisomerase I. On lane 7 nuclear proteins that did not adsorb to PBA-30 were applied. A single band at approximately 95 kDa is visible, which corresponds to topoisomerase I (Guldner et al., 1986) and attests to the specificity of the antibody. Three bands at 120, 95, and 85 kDa are discernible for the PBA-30 adsorbed proteins. The intensities of the first two bands increased upon exposure to AO (lanes 2 and 4). While the 95-kDa band undoubtedly corresponds to topoisomerase I, the identity of the 120-kDa band remains unknown. The latter band is not ADPR-transferase since the antibody used in our work reportedly does not cross-react with this enzyme (G. de Murcia, personal communication). The intensity of the 85-kDa band remained constant in all samples, and it appears likely that it belongs to a non-ADP-ribosylated cross-reacting protein that is unspecifically retained on the PBA-30 column. The addition of benzamide (100 μM) reduced the AO-induced increases in the 120- and 95-kDa bands (lanes 3 and 5). As was the case for the immunoblots with anti-ADPR-transferase antibody, the densitometer readings

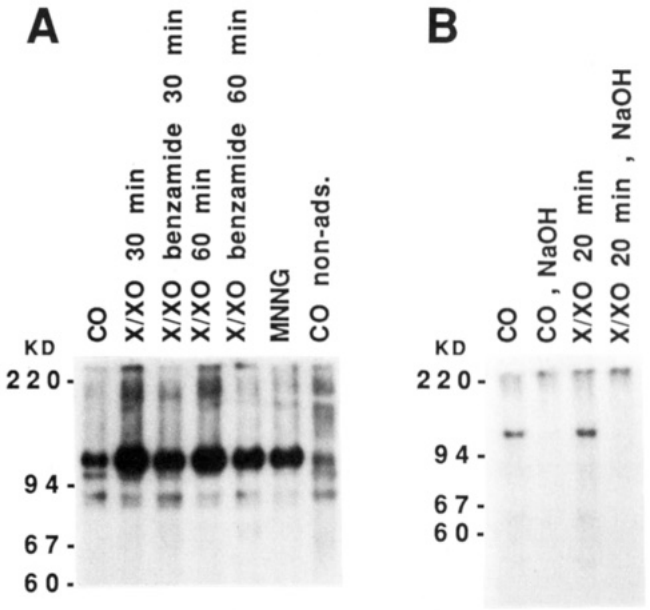

FIGURE 2: ADPR-transferase immunoblots of acid-insoluble nuclear material from JB6 (clone 41) cells that had been treated with active oxygen or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Experimental conditions were as described in the legend to Figure 1 and under Methods. A polyclonal rabbit anti-ADPR-transferase antibody was used and reacted with ^{125}I-labeled donkey anti-rabbit Ig for autofluorography. (A) Induction of poly(ADP-ribosylation) by active oxygen and MNNG, respectively, and effect of the ADPR-transferase inhibitor benzamide (100 μM). (B) Removal of poly(ADPR) chains from ADPR-transferase by treatment of nuclear preparations with 0.1 N NaOH as described under Methods.

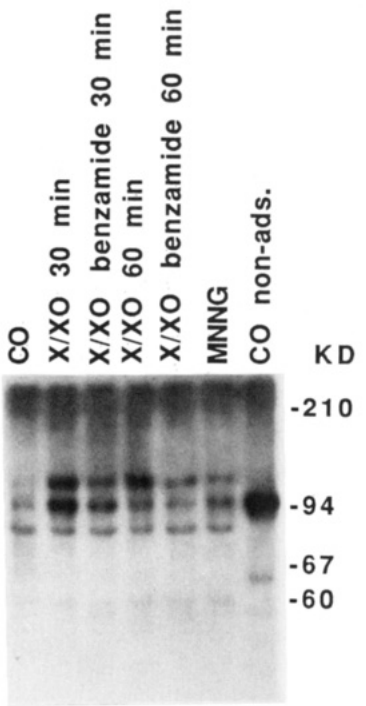

FIGURE 3: Topoisomerase I immunoblots of acid-insoluble nuclear material from JB6 (clone 41) cells that had been treated with active oxygen or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Experimental conditions were as described in the legend to Figure 1 and under Methods. A polyclonal rabbit anti-topoisomerase I antibody was used and reacted with ^{125}I-labeled donkey anti-rabbit Ig for autofluorography.

for the 95-kDa band were directly proportional to the amounts of [^{3}H]leucine protein radioactivity applied on the gel (see Figure 1B), allowing the calculation of the extent of poly-

Table II: Poly(ADP-ribosylation) of Topoisomerase I by Active Oxygen (JB6 Clone 41)

	normalized densitometer readings[b]	poly(ADP-ribosylation) of topoisomerase I (%)
adsorbed on boronate[a]		
control	4.2	0.11
X/XO, 30 min	25.5	0.68
X/XO + benzamide, 30 min	10.8	0.29
X/XO, 60 min	5.0	0.13
X/XO + benzamide, 60 min	3.6	0.10
MNNG, 20 min	9.3	0.25
nonadsorbed on boronate control	3704	

[a] Cells exposed to active oxygen (X/XO 50/5 μg/mL), active oxygen plus benzamide (100 μM), or MNNG (5 μg/mL) for the indicated lengths of time. [b] Densitometer readings of the 95-kDa band from Western blots normalized to 1% of the total nuclear proteins from 1.4 $\times$ 10^8 cells.

(ADPR) substitution of topoisomerase I. From the data listed in Table II, it is evident that the extent of steady-state substitution of topoisomerase I with poly(ADPR) is low (0.11%), that AO treatment caused a 6-fold increase within 30 min, and that the level of substitution has essentially returned to control levels within 60 min.

Discussion

In general, it has not been possible to selectively radioactively label the cellular NAD pool with ^{32}P in intact cells. While labeling of NAD with [^{3}H]adenosine (Aldolph & Song, 1985a,b) or [^{3}H]adenine (Cardenas-Corona et al., 1987) has been successful, the radioactivity associated with many poly(ADPR) acceptor proteins is insufficient for fluorometry. This represents a major obstacle for studies of the poly(ADP-ribosylation) of nuclear proteins in vivo. The experimental strategy combining phenylboronate PBA-30 affinity chromatography with immunoblots taken in the present work circumvents this difficulty. Since phenylboronate retains mono- and poly(ADP-ribosylated) proteins, our data do not yield information about the chain lengths of the substituents. However, since nuclear proteins were isolated mono(ADP-ribosylated) proteins probably result from the degradation of poly(ADPR) chains rather than bona fide ADP-ribosylation (Althaus & Richter, 1987; Althaus et al., 1985). The combination with immunoblots is necessary because of the limited specificity of the phenylboronate PBA-30 column. From our results with the antibody against poly(ADPR) chains (Figure 1), it is evident that PBA-30 effectively removed poly(ADP-ribosylated) proteins because no bands were visible in the nonadsorbed flow-through. However, PBA-30 in addition also retained non-poly(ADP-ribosylated) proteins. This is evident from the observation that the fraction of proteins retained always represented 1.3–1.5% of the total and did not increase upon treatment with inducers of poly(ADPR) synthesis. Therefore, retention on PBA-30 does not suffice for the identification of poly(ADPR) acceptor proteins. We used the following additional criteria: (1) detection by poly(ADPR) antibodies on immunoblots; (2) alkaline hydrolysis of the poly(ADPR) substituents; (3) increase in specific bands on immunoblots with antibodies against specific acceptor proteins; (4) inhibition of the reaction by the ADPR-transferase inhibitor benzamide. To avoid undesirable side reactions, low benzamide concentrations (100 μM) were used. Even under these conditions poly(ADP-ribosylation) was inhibited by more than 50%.

The 120-kDa band on the blots with poly(ADPR) antibodies (Figure 2A) undoubtedly represents ADPR-transferase (Adamietz & Rudolph, 1984; Kawaichi et al., 1981; Jump & Smulson, 1980; Singh et al., 1985), and for this case the first two criteria listed above are fulfilled. ADPR-transferase is the only non-histone nuclear protein that is sufficiently constitutively substituted with poly(ADPR) (i.e., estimated at 4% from immunoblots by using antibodies against ADPR-transferase; see Table I) to give rise to a distinct band in untreated control cultures. We estimate that the limit of detection with the poly(ADPR) antibody available to use lies at about 1% poly(ADPR) substitution. Distinct bands at 110, 85, and 73 kDa are visible on the blots of the treated samples, but their identity is not known. Since these bands are not visible on the blots with antibodies against either ADPR-transferase or topoisomerase I, they are not due to proteolytic degradation of these proteins as observed in previous work. The diffuse bands below 35 kDa on the poly(ADPR) blots probably correspond to histones that are coprecipitated with the acid-insoluble nuclear proteins. Interestingly, these bands move to slightly higher molecular weights following treatment with MNNG than with AO, suggesting that histones under the latter conditions are substituted with shorter poly(ADPR) chains. An account of histone poly(ADP-ribosylation) in intact human epithelial cells in response to AO and MNNG will be published separately. With this exception no striking qualitative differences are discernible for the pattern of acceptor proteins induced by AO relative to MNNG on any of the blots with the three antibodies used in this work. No simple correlation is expected for the intensities of the bands on Western blots with antibodies against poly(ADPR) chains and individual acceptor proteins, respectively. The former depend on the poly(ADPR) chain length and extent of substitution, the latter on the amounts of protein.

According to previous measurements with the fluorescence method, total AO-induced poly(ADPR) in JB6 clone 41 cells remained substantially elevated above control levels 1 and 2 h after treatment (Muehlematter et al., 1988). Similarly, in the present work with antibodies against ADPR-transferase, 25% of the enzyme molecules (relative to 4% in the untreated controls, see Table I) were still ADP-ribosylated after 60 min. This result is supported by blots using the antibody directed against poly(ADPR) (Figure 1). Indeed, because ADPR-transferase appears to represent the predominant acceptor in several cell systems (Singh et al., 1985; Adamietz, 1987), it is likely that measurements of total poly(ADPR) mostly reflect the substitution of this enzyme. The stability of poly(ADPR) substitution varies for different acceptors. While the level of poly(ADP-ribosylation) of the 95-kDa topoisomerase I band returned to control values within 60 min, the poly(ADPR) substitution of the unidentified 120-kDa protein on the same blot (Figure 3) slightly increased from 1 to 2 h after treatment.

Our results indicate extensive auto-poly(ADPR) of ADPR-transferase in response to AO. This presumably results in the inactivation of the enzyme (Althaus & Richter, 1987; Ferro & Olivera, 1982). However, conjugation with short poly(ADPR) chains in response to the alkylating agent dimethyl sulfate did not inactivate the enzyme (Adamietz, 1987). If poly(ADPR) occurs preferentially at proteins in the neighborhood of DNA strand breakage, the observed level of poly(ADPR) substitution may well suffice to locally inactivate the enzyme. It is tempting to speculate that the autoinactivation of ADPR-transferase is part of a feedback circuit with the function to avoid excessive poly(ADPR) and NAD and ATP depletion.

Topoisomerase I plays a crucial role in DNA replication and possibly other processes of DNA metabolism. It transiently incises one DNA strand (Wang, 1981, 1987), acts as a swivel for the unwinding of the parental DNA strand (Yang et al., 1987), propagates the replication fork (Wang, 1987), and participates in the segregation of the progeny molecules (Minden & Marian, 1986). Inactivation of topoisomerase I by poly(ADP-ribosylation) (Ferro et al., 1983; Ferro & Olivera, 1984; Jongstra-Bilen, 1983) in the neighborhood of DNA strand breakage could have the function to locally shut down DNA replication until repair has occurred (Ferro et al., 1984). However, as observed for ADPR-transferase, short poly(ADPR) chains did not inactivate the enzyme (Adamietz, 1987).

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Time-Resolved Fluorescence Spectroscopy of Human Adenosine Deaminase: Effects of Enzyme Inhibitors on Protein Conformation†

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ABSTRACT: Adenosine deaminase, a purine salvage enzyme essential for immune competence, was studied by time-resolved fluorescence spectroscopy. The heterogeneous emission from this four-tryptophan protein was separated into three lifetime components: τ_1 = 1 ns and τ_2 = 2.2 ns with an emission maximum at about 330 nm and τ_3 = 6.3 ns with emission maximum at about 340 nm. Solvent accessibility of the tryptophan emission was probed with polar and nonpolar fluorescence quenchers. Acrylamide, iodide, and trichloroethanol quenched emission from all three components. Acrylamide quenching caused a blue shift in the decay-associated spectrum of component 3. The ground-state analogue enzyme inhibitor purine riboside quenched emission associated with component 2 whereas the transition-state analogue inhibitor deoxycoformycin quenched emission from both components 2 and 3. The quenching due to inhibitor binding had no effect on the lifetimes or emission maxima of the decay-associated spectra. These observations can be explained by a simple model of four tryptophan environments. Quenching studies of the enzyme–inhibitor complexes indicate that adenosine deaminase undergoes different protein conformation changes upon binding of ground- and transition-state analogue inhibitors. The results are consistent with localized structural alterations in the enzyme.

Adenosine deaminase (EC 3.5.4.4), a purine salvage enzyme, catalyzes the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. Hereditary deficiency of adenosine deaminase is associated with a severe combined immunodeficiency (Giblett et al., 1972) which typically results in severe lymphopenia. The specific lympholytic effect of adenosine deaminase inhibitors has been exploited in the use of these compounds for chemotherapy, especially for certain leukemias that are associated with a marked increase in lymphocytes.

The structures of three adenosine deaminase inhibitors are shown in Figure 1. Ground-state or substrate analogue inhibitors, which include purine riboside, exhibit fast binding kinetics, resulting in instantaneous inhibition (Frieden et al., 1980). Transition-state analogue inhibitors, which resemble the proposed tetrahedral intermediate in the reaction catalyzed by adenosine deaminase, include 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR)[1] and the antibiotics coformycin and deoxycoformycin (Evans & Wolfenden, 1970; Agarwal et al., 1978). These inhibitors exhibit slow binding kinetics (Frieden et al., 1980). It is postulated that the slow binding kinetics are due to conformational changes in the protein, which result in formation of the transition-state complex. The rearrangements in the protein may occur in regions distant from the site. Alternatively, they may be confined to structural changes around the active site. Another potent inhibitor of adenosine deaminase, *erythro*-9-(hydroxy-3-nonyl)adenine (EHNA), which is classified as a competitive, ground-state analogue inhibitor, exhibits semislow binding kinetics (Frieden et al., 1980). Because EHNA is not deaminated by the enzyme, it has been proposed that EHNA binds at an auxiliary binding region near the active site (Woo

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[1] Abbreviations: EHNA, *erythro*-9-(hydroxy-3-nonyl)adenine; DHMPR, 1,6-dihydro-6-(hydroxymethyl)purine riboside; NATA, *N*-acetyltryptophanamide; PBS, phosphate-buffered saline; TCE, trichloroethanol.

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