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Macromolecular association of ADP-ribosyltransferase and its correlation with enzymic activity

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The macromolecular self-association of ADP-ribosyltransferase protein in solution was studied by several experimental techniques: quantitative gel filtration, electrophoretic analyses in non-denaturing gels, and cross-linking the enzyme protein with glutaraldehyde, dimethyl pimelimidate, dimethyl suberimidate, dimethyl 3,3′-dithiobisproprionimidate and tetranitromethane. The self-association of the polypeptide. components obtained by plasmin digestion was also determined by using the above cross-linking agents. Monomers and cross-linked dimers of the enzyme protein, possessing enzymic activity, were separated in non-denaturing gels by electrophoresis. The basic polypeptide fragments, exhibiting molecular masses of 29 kDa and 36 kDa, self-associated, whereas the polypeptides with molecular masses of 56 kDa and 42 kDa associated only to a negligible extent, indicating that the peptide regions that also bind DNA and histones are probable sites of self-association in the intact enzyme molecule. Macromolecular association of the enzyme was indicated by a protein-concentration-dependent red-shift in protein fluorescence. The specific enzymic activity of the isolated ADP-ribosyltransferase depended on the concentration of the enzyme protein, and at 2.00 μM concentration the enzyme was self-inhibitory. Dilution of the enzyme protein to 30–40 nM resulted in a large increase in its specific activity. Further dilution to 1–3 nM coincided with a marked decrease of specific activity. Direct enzymic assays of electrophoretically separated monomers and cross-linked dimers demonstrated that the dimer appears to be the active molecular species that catalyses poly(ADP-ribose) synthesis. The NAD^+ glycohydrolase activity of the enzyme was also dependent on protein concentration and was highest at 1–3 nM enzyme concentration, when polymerase activity was minimal, indicating that the monomeric enzyme behaved as a glycohydrolase, whereas poly(ADP-ribosyl)ation of enzyme molecules was maximal when the enzyme tends to be self-associated to the dimeric form.

INTRODUCTION

ADP-ribosyltransferase (ADPRT, EC 2.4.2.30) is a specific DNA-binding nuclear protein of higher eukaryotes that consists of a single polypeptide with a molecular mass of 116–120 kDa, as determined by SDS/PAGE in numerous laboratories (cf. Mandel *et al.*, 1982; Ueda & Hayaishi, 1985; Gaal & Pearson, 1985; Althaus & Richter, 1987; Buki *et al.*, 1987). Some uncertainties regarding the molecular mass of ADPRT as detected by gel filtration and ultracentrifugation have been reported (Ushiro *et al.*, 1987; Sastry & Kun, 1988). Abnormal sedimentation coefficients, originally observed by Kristensen & Holtlund (1978), were explained by presumed anomalous symmetry of the protein molecule. Alternatively, apparently unusual macromolecular properties may be due to an association of ADPRT molecules, implying also the possibility that more than one ADPRT molecule could participate in the enzymic reaction of auto-poly(ADP-ribosyl)ation of the enzyme. This question has been indirectly addressed by Ikejima *et al.* (1987), who concluded from rate measurements that the polymerase catalysis appears to be monomolecular with respect to the ADPRT molecule. However, customary tests for ADPRT activity consisting of the quantification by radiochemical analysis of ADP-ribose residues covalently bound to ADPRT cannot distinguish between chain-initiator adducts and possible secondary polymer protein adducts that may be formed by trans-(ADP-ribosyl)ation to another molecule(s) of ADPRT (Bauer & Kun, 1985; Bauer *et al.*, 1986). Furthermore it was shown by Honegger *et al.* (1989) that, if an enzyme auto-modifies itself, kinetic analysis as a function of protein concentration cannot discriminate between unimolecular and bimolecular mechanisms. For these reasons the participation of one or two enzyme molecules in auto-poly(ADP-ribosyl)ation is an open question. Since the detailed molecular mechanisms of poly(ADP-ribosyl)ation are as yet unclear, it is of importance to identify component reactions of the enzymic transfer of ADP-ribose from NAD^+ to the enzyme, macromolecular association of the enzyme being one of them. Macromolecular association of ADPRT assumes further significance in the light of the recently identified second molecular activity of ADPRT, exerted on DNA topology (Sastry & Kun, 1988; Sastry *et al.*, 1989), which occurs in the absence of NAD^+, and is actually self-regulated by NAD^+ binding. The present paper is concerned with the analysis of self-association of ADPRT by chromatographic, electrophoretic and protein-cross-linking techniques. The dependence of the rates of both auto-modification of the enzyme by ADPR and the NAD^+ glycohydrolase activity of ADPRT on protein concentration indicated a critical role of self-association in the regulation of the catalytic activities of this protein.

EXPERIMENTAL

Preparation of ADPRT and its peptides

More than 95% homogeneous ADPRT and its peptides were isolated as described previously (Buki *et al.*, 1987; Buki & Kun, 1988).

Abbreviations used: ADPRT, ADP-ribosyltransferase; Capso, 3-cyclohexylamino-2-hydroxy-1-propanesulphonic acid; PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}; NEPHGE, non-equilibrium pH-gradient electrophoresis.

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Gel-permeation chromatography

This was performed with a TSK-SW-3000 column, connected to a Beckman 421 h.p.l.c. controller unit, two Beckman 100A h.p.l.c. pumps and a Hitachi u.v. detector unit. The injected polypeptides were eluted with 0.5 M-sodium acetate buffer, pH 5.0, with a flow rate of 0.93 ml/min. Determination of the effect of dilution of ADPRT solutions on the apparent molecular mass of the enzyme, as assayed by gel-permeation chromatography, required the development of an immunochemical microtest for ADPRT that far exceeded the sensitivity of u.v. monitoring of the protein, the latter technique not being applicable to dilute solutions employed in experiments shown in Fig. 1(*c*). Samples (5 or 50 μl) from fractions collected from TSK 3000 column eluates (Figs. 1*b* and 1*c* respectively) were dried into wells of Costar plates at 4 °C for 48 h, then washed twice with 200 μl portions of washing buffer (PBS containing 0.05% BSA, 0.05% Tween 20 and 0.1 mg of Thimerosal/l). Non-specific binding sites were occluded with 200 μl of blocking buffer, consisting of PBS containing 1% BSA and 0.1% NaN_3, by incubation for 30 min at room temperature, followed by washing twice with 200 μl portions of washing buffer and the addition of 50 μl of anti-ADPRT polyclonal antibody [rabbit IgG, raised against calf thymus ADPRT (cf. Buki *et al.*, 1987), diluted 1:5000 in the washing buffer]. Incubation with the antibody lasted 4 h at room temperature. The wells were then washed with three successive 200 μl portions of washing buffer, followed by the addition of 50 μl of 1:1000-diluted (in washing buffer) horseradish-peroxidase-labelled goat anti-(rabbit IgG) antibody (Sigma Chemical Co.), and the mixture was further incubated for 1 h at room temperature. The wells were then washed four times with 200 μl portions of washing buffer and once with substrate buffer (0.1 M-citrate buffer, pH 4.5). To each well 100 μl of a solution of peroxidase substrate was added, consisting of 4 mg of *o*-phenylenediamine and 4 μl of 30% (v/v) H_2O_2 freshly dissolved into 10 ml of substrate buffer, and the contents of the wells were incubated for 20 min at room temperature. The peroxidase reaction was terminated by 10 μl of 6 M-HCl added to each well and the absorbance of the chromogen was determined at 495 nm.

Electrophoresis in non-denaturing gels

The running buffer was 20 mM-Capso/NaOH buffer, pH 10.3, containing 10 mM-2-mercaptoethanol. This high pH was necessary to permit entry of the enzyme into the gel. The stacking gel was 4% polyacrylamide (the acrylamide/bisacrylamide ratio was 37.7:2.3 throughout), dissolved in the above running buffer. The separating gel consisted of 5–8% acrylamide, dissolved in 100 mM-Capso/NaOH buffer, pH 10.3, containing 10 mM-2-mercaptoethanol. In some instances a linear acrylamide gradient between 5 and 15% was employed. Electrophoresis was performed at 100 V for 4–6 h, with the exception that this time was extended to 8 h for gradient gels. Polypeptide samples (10–40 μg/50 μl) were mixed with an equal volume of a solution composed of 40 mM-Capso/NaOH buffer, pH 10.3, containing 20 mM-2-mercaptoethanol, 20% (v/v) glycerol and 0.02% Bromophenol Blue. Immunostaining was carried out as reported previously (Buki *et al.*, 1987) by soaking gels in 20 mM-Capso/NaOH buffer, pH 10.3, containing 20 mM-2-mercaptoethanol for 10 min, followed by transblotting in 20 mM-Capso/NaOH buffer, pH 10.3, containing 20% (v/v) methanol to nitrocellulose membranes for 3 h at 4 °C.

Electrophoresis in two dimensions

A 10 μg portion of ADPRT was first exposed to electrophoresis on a 5–15%-gradient non-denaturing gel as described above, and after the tracking dye had reached the bottom of the gel this gel segment was vertically sliced off and immersed for 60 min in the SDS-containing stacking gel buffer (Laemmli, 1970), which was changed twice within 60 min. The gel slice was then exposed to electrophoresis in the second dimension by placing it horizontally on top of a 10% separating gel containing SDS. The two gels were connected by a thin layer of 1% agarose, dissolved in the stacking buffer. Electrophoresis was performed at 100 V for 3–4 h, and the gel was fixed with 20% (w/v) trichloroacetic acid and stained with Coomassie Blue, and then de-stained in 10% (v/v) acetic acid and dried.

Determination of the molecular mass of ADPRT in non-denaturing gels

This was performed in accordance with Ferguson (1964) and Chambrach & Rodbard (1971) as follows. A 15 μg portion of enzyme protein was run in 5%, 6%, 7% and 8% acrylamide gels under non-denaturing conditions together with marker proteins (see the legend to Fig. 3). After Coomassie Blue staining, R_F values for all the polypeptides were calculated from the gel and the $100 \times \log(R_F \times 100)$ values were plotted against the concentration (%) of acrylamide. From the plots thus obtained the absolute values of slopes were replotted on double-logarithmic paper against the molecular masses of known proteins serving as molecular standards (Fig. 4*b*).

Cross-linking with glutaraldehyde

Application of this cross-linking agent was based on the reaction mechanism described by Richards & Knowles (1968). ADPRT protein and its polypeptide fragments were diluted to desired concentrations (between 0.3 and 1.6 mg/ml) with 0.1 M-triethanolamine/HCl buffer, pH 8.0, glutaraldehyde was added to a final concentration between 1.3 and 2.5 mM and the mixture was incubated for 10 min at room temperature. The reaction was terminated by quenching with a 4-fold molar excess of hydrazine sulphate, pH 8.0, followed by an equal volume of SDS sample buffer (Laemmli, 1970), and the mixture was further incubated for 15 min. Portions (up to 100 μl each) were then loaded on to either 10% (for polypeptides) or 6% (for ADPRT) acrylamide gels (Laemmli, 1970) and developed as described above. Hydrogen-bonded association of polypeptides was disrupted by pretreatment with 50 mM-SDS (Hermann *et al.*, 1979). Coomassie Blue-stained peptide bands were quantified as described previously (Buki & Kun, 1988).

Cross-linking of ADPRT and of polypeptides derived from ADPRT by plasmin digestion with bifunctional imidoesters

The general procedures of Lutz *et al.* (1977) were followed. ADPRT or its polypeptides (20 μg) were dissolved in 20 μl of 0.1 M-triethanolamine/HCl buffer, pH 8.0, and the samples were incubated with cross-linking reagents at a final cross-linker concentration of 35 mM (either dimethyl suberimidate, dimethyl pimelimidate or dimethyl 3,3′-dithiobispropionimidate) for 20 min at 23 °C. Hydroxylamine (final concn. 0.1 M) was then added (5 μl) for quenching and 25 μl of Laemmli (1970) sample buffer, from which reducing agents were omitted, and portions containing between 1 and 10 μg of polypeptides were exposed to SDS/PAGE in 8% Laemmli (1970) gels for 2.3–3 h. When disulphides were reduced, 2-mercaptoethanol at a final concentration of 1% (v/v) was applied for 10 min. The quantity of cross-linked polypeptides was determined by colorimetric assay of protein-bound Coomassie Blue extracted from gel slices (Buki & Kun, 1988).

Cross-linking of ADPRT or its polypeptides with tetranitromethane

This was performed by the procedure of Martinson &

McCarthy (1975). ADPRT or its polypeptides were dissolved in 20 μl of 100 mM-triethanolamine/HCl buffer, pH 8.0. Then 1 μl of 1% (v/v) tetranitromethane solution in ethanol was added to yield a final concentration of 2–2.5 mM, and the mixture was incubated for 20 min at 23 °C. Finally, 2-mercaptoethanol was added to a final concentration of 2–2.5 mM to remove the cross-linker, and the incubation was continued for 10 min. After the samples had been mixed with an equal volume of Laemmli sample buffer, portions containing 1 μg of protein were loaded on to SDS/10%-PAGE gels. Proteins were detected by the silver staining method of Merrill *et al.* (1980).

Determination of enzymic activities of monomers and glutaraldehyde-cross-linked dimers

This was carried out after their separation by non-equilibrium pH-gradient electrophoresis (NEPHGE) in accordance with O'Farrell *et al.* (1977) as described by Young *et al.* (1983) as follows. A 12 μg portion of ADPRT and 5 μg of the synthetic DNA analogue (the octameric duplex C; cf. Hakam *et al.*, 1987) were preincubated for 2 min in 100 mM-triethanolamine/HCl buffer, pH 8.0, then treated with glutaraldehyde (2.4 mM) for 10 min at room temperature in a volume of 20 μl. At the end of the incubation 5 μl of aqueous hydrazine sulphate solution (to achieve 20 mM final concentration) was added to quench the unused glutaraldehyde. In control tests hydrazine sulphate was added before the glutaraldehyde. Then 1 μl of Ampholine (pH 3–10) was added and the mixture (20 μl) was loaded on to a NEPHGE gel. The gel contained 6% polyacrylamide and 2% of Ampholine (pH 3–10). The upper buffer tank contained 55 mM-H_3PO_4. In this system polypeptides migrated towards the cathode submerged in 25 mM-NaOH. Coloured protein standards (Bio-Rad Laboratories standards for isoelectric focusing) were used to monitor polypeptide migration. When the most basic protein (cytochrome *c*) was 8 cm from the start line the electrophoretic process was terminated, and the gel was sliced and incubated successively in 20 ml of 0.1 M-Tris/HCl buffer, pH 8.0, for 20 min, then in 10 ml of the same buffer supplemented with 10 mM-$MgCl_2$, 7 mM-2-mercaptoethanol and 500 ng of octameric duplex C/ml. The substrate [^{32}P]NAD$^+$ (50 μCi; 1 μM) was then added and incubation was continued for 45 min at 23 °C. At the end of the incubation the gel was fixed in 20% (w/v) trichloroacetic acid overnight, then washed first with 10% (w/v) trichloroacetic acid followed by a solution of 20% (v/v) acetic acid containing 20% (v/v) ethanol, stained with Coomassie Blue, de-stained, dried, and autoradiographed for 2 h. The identity of ADPRT as a monomer or dimer in the NEPHGE gel was confirmed in a two-dimensional system consisting of NEPHGE in the first and SDS/10%-PAGE in the second dimension. The location of the Coomassie Blue-stained proteins on the NEPHGE gel and the black spots of the autoradiogram coincided. The gel was cut into 5 mm-thick slices, and the radioactivity was determined by scintillation spectrometry and the quantity of Coomassie-stained proteins by spectrophotometry (Buki & Kun, 1988).

Quantitative separation of monomers of ADPRT from glutaraldehyde-cross-linked dimers

This was performed in NEPHGE gels containing 6 M-urea by the method described above. Urea aided the quantitative separation of the two species (Fig. 5), whereas without urea, when 70% of enzymic activity was preserved, some contamination of monomers by dimers was technically unavoidable (Table 3).

Assay of ADPRT activity

The enzymic activity of ADPRT was assayed by techniques described previously (Buki *et al.*, 1987; Hakam *et al.*, 1987) adapted to specific conditions.

Protein fluorescence (Lakowicz, 1983; Fleischmann *et al.*, 1988) was determined in microcuvettes of 45 μl volume.

Assay of NAD$^+$ glycohydrolase activity

NAD$^+$ glycohydrolase activity was assayed in ADPRT solutions diluted as shown in Tables 2 and 4 by the determination by h.p.l.c. of free ADP-ribose (Hakam & Kun, 1985) formed after incubation of ADPRT with NAD$^+$.

RESULTS

Chromatographic evidence for the self-association of ADPRT and of basic polypeptides obtained by plasmin digestion

The ADPRT protein separated into two elution peaks on the TSK-SW-3000 column, one with an apparent molecular mass of slightly over 200 kDa, and one with the known molecular mass of 116–120 kDa. Both protein peaks gave identical bands on SDS/PAGE, consistent with a molecular mass of 116–120 kDa (Fig. 1*a* inset). As shown in Figs. 1(*a*) and 1(*b*), u.v. monitoring

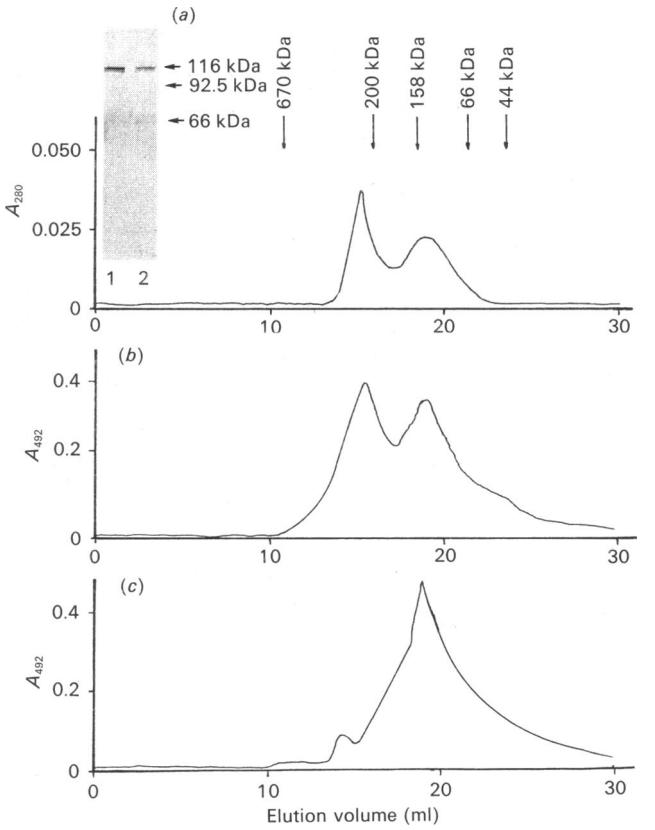

Fig. 1. Gel-permeation chromatography of ADPRT on a TSK-SW-3000 column

Portions [25 μg (*a* and *b*) or 2.5 μg (*c*)] of ADPRT, dissolved in 20 μl of the elution buffer (0.5 M-sodium acetate buffer, pH 5.0) were injected on to the TSK-SW-3000 column (see the Experimental section), and h.p.l.c. separation at a flow rate of 0.93 ml/min and collection of 0.4 ml fractions were carried out. In (*a*) elution of peaks was monitored by the u.v. detector. In (*b*) and (*c*) ADPRT protein was determined by e.l.i.s.a. Since (*b*) contained 10 times more enzyme than (*c*), 5 μl portions of (*b*) and 50 μl portions of (*c*) were assayed by the colorimetric immuno-test (see the Experimental section). The inset in (*a*) is a Coomassie Blue-stained SDS/PAGE gel, representing samples removed from each peak separately, eluted at 18.3 min and 20 min respectively. Lanes 1 and 2 indicate elution peaks 1 and 2. The scale next to the gels shows the positions of molecular-mass markers. Molecular-mass markers, shown at the top of panel (*a*) were thyroglobulin (670 kDa), amylase (200 kDa), IgG (158 kDa), BSA (66 kDa) and ovalbumin (44 kDa).

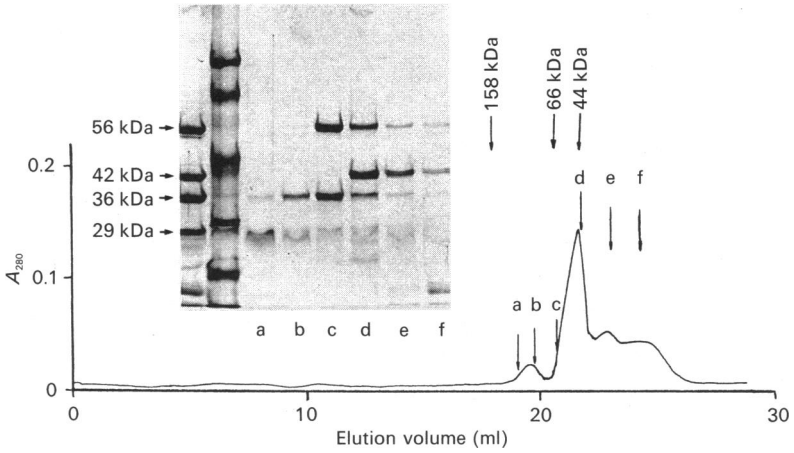

Fig. 2. Gel-permeation chromatography of polypeptides prepared by plasmin digestion of ADPRT on a TSK-SW-300 column

Polypeptides, obtained from a digestion of 50 μg of ADPRT, were injected in 50 μl of elution buffer and developed as described in the legend to Fig. 1. Both u.v. absorption of emerging polypeptide peaks and their polypeptide composition, determined on 20 μl portions by SDS/PAGE (inset), are shown, and correspondence between u.v.-absorption peaks and their composition is indicated by corresponding letters a–f. The first lane (left) in the SDS/PAGE inset illustrates polypeptides present in the unfractionated plasmin digest, and the second lane shows molecular-mass standards (phosphorylase *b*, 92.5 kDa; BSA, 66 kDa; ovalbumin, 44 kDa; soy-bean trypsin inhibitor, 21 kDa), and peptides are shown in lanes a–f, corresponding to elution peaks a–f.

and immunodetection techniques gave identical results. A 10-fold dilution of the ADPRT solution nearly completely abolished the appearance of the peak corresponding to 200 kDa (Fig. 1*c*), suggesting that dilution of the enzyme protein tends to dissociate the dimeric form to monomers.

Not only the ADPRT molecule but two of its basic polypeptide components obtained by plasmin digestion (Buki & Kun, 1988) exhibited self-association, as tested by the gel-filtration method. As shown in Fig. 2, gel filtration of the polypeptides of 29 kDa and 36 kDa, but not of the polypeptides of 42 kDa and 56 kDa, indicated self-association, a conclusion based on SDS/PAGE analysis of the polypeptides of ADPRT fractionated on the TSK-SW-3000 column (Fig. 2). According to molecular-mass standards the polypeptide of molecular mass 29 kDa was eluted at a position corresponding to an apparent molecular mass higher than 66 kDa (compare fraction a in the elution profile with gel position), and a similar apparent aggregation was seen with the polypeptide of molecular mass 36 kDa. On the other hand the polypeptides of molecular mass 56 kDa and 42 kDa respectively appearing in fractions c and d more closely corresponded to the molecular masses of these peptides obtained by direct digestion of ADPRT by plasmin (Buki & Kun, 1988), as deduced from comparison with standards. However, molecular-mass assays based on molecular filtration alone may be uncertain because of the possible artifacts inherent in this technique (Regnier, 1983).

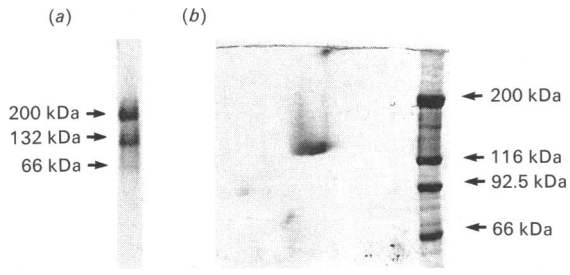

Fig. 3. Electrophoretic separation of ADPRT in a non-denaturing gel and re-electrophoresis in the second dimension by SDS/PAGE

A 10 μg portion of ADPRT was subjected to electrophoresis on a 5–15 %-linear-gradient non-denaturing polyacrylamide gel (*a*) for 8 h together with molecular-mass markers, which were: amylase (200 kDa), BSA dimer (132 kDa) and BSA monomer (66 kDa) (standards are not shown). The lane shown in (*a*) was excised and subjected to re-electrophoresis by 10 %-acrylamide SDS/PAGE in the second dimension (see the Experimental section) as shown in (*b*). The lane at the right end of (*b*) shows molecular-mass markers in SDS/PAGE (second dimension): myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (92.5 kDa) and BSA (66 kDa). Polypeptide bands were stained with Coomassie Blue.

Electrophoretic separation of self-associated forms of ADPRT in non-denaturing gels

When the ADPRT protein was first exposed to electrophoresis in non-denaturing gels (see the Experimental section) dimerization was indicated by the appearance of monomeric and dimeric molecular species (Fig. 3*a*). However, the two protein bands, when re-electrophoresed in an SDS-containing gel (Fig. 3*b*), tended to fuse mainly to protein bands corresponding to approx. 120–130 kDa molecular mass. There is some distortion in the estimation of molecular masses because the protein standards (right-hand lane) were exposed to direct SDS/PAGE without prior electrophoresis in the non-denaturing gel.

Electrophoretic analysis of apparent molecular masses of ADPRT and its polypeptides obtained by plasmin digestion in non-denaturing gels

It is apparent from Fig. 4(*a*) that in an 8 % non-denaturing gel ADPRT and the polypeptides of 29 kDa and 36 kDa exhibited associative behaviour, ADPRT corresponding to a molecular mass of approx. 200 kDa, the 29 kDa polypeptide to about 100 kDa and the 36 kDa polypeptide to slightly above 100 kDa (Fig. 4*a*). Fig. 4(*b*) represents a Ferguson plot where the ordinate is the negative slope of the plots of $100 \times \log(R_F \times 100)$ versus concentration (%) of acrylamide and the abscissa is the apparent molecular mass of ADPRT and of marker polypeptides. Here again the apparent molecular mass of ADPRT corresponded to 200 kDa (see dashed co-ordinates).

In the above experiment the identity of ADPRT was established by immunostaining in Western blots as described in the Experimental section (results not shown).

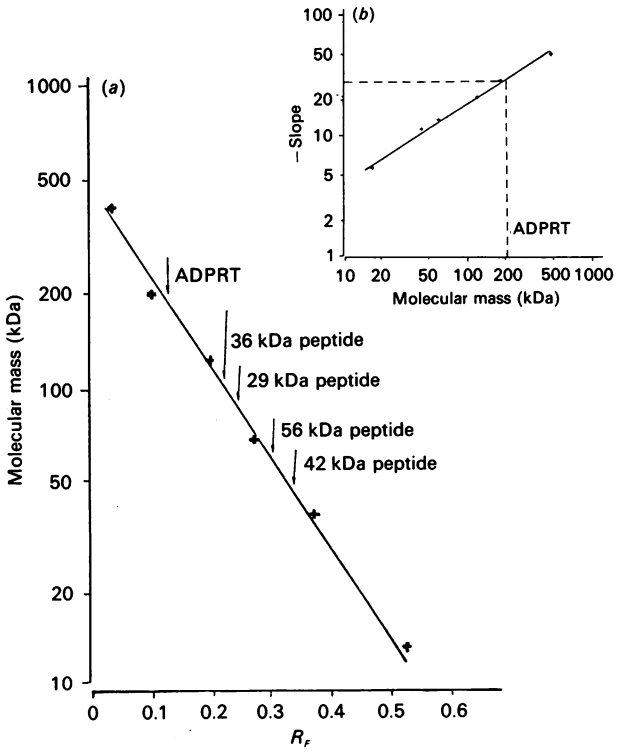

Fig. 4. Determination of the apparent molecular masses of ADPRT and its polypeptides obtained by digestion with plasmin by electrophoresis in non-denaturing gels as illustrated by the Ferguson method

(*a*) A 10 μg portion of ADPRT, 9 μg of 29 kDa polypeptide, 12 μg of 36 kDa polypeptide and 16 μg of a mixture of equal amounts of 42 kDa and 56 kDa polypeptides, each in separate lanes, were subjected to electrophoresis on an 8% polyacrylamide gel under non-denaturing conditions (see the Experimental section) for 4 h. R_F values (abscissa) were calculated and the apparent molecular-mass values can be read on the ordinate and compared with the known molecular masses of the polypeptides. (*b*) Portions (15 μg) of ADPRT were subjected to electrophoresis in 5%, 6%, 7% and 8% acrylamide gels under non-denaturing conditions (see the Experimental section) simultaneously with the molecular-mass markers apoferritin (443 kDa), β-amylase (200 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa), ovalbumin (44 kDa) and lactalbumin (17 kDa) as indicated by points on the line drawn in the Figure. The ordinate scale is the negative slopes of lines obtained by plotting $100 \times (R_F \times 100)$ for each polypeptide against the percentage concentration of acrylamide. The abscissa scale is the molecular-mass values of the polypeptides. The plot is on a double logarithmic scale.

Cross-linking of ADPRT and its polypeptides produced by plasmin digestion

Further evidence for associative behaviour of the polypeptides was obtained by cross-linking of ADPRT and its polypeptide components with glutaraldehyde in aqueous solution. It is known that glutaraldehyde in solution is present as a polymer that forms cross-linked Michael adducts with polypeptides (Richards & Knowles, 1968). The degree of cross-linking is a function of the concentrations of both macromolecules and glutaraldehyde, and therefore the quantity of cross-linked molecules indicates equilibria, as described also for histones (Olins & Wright, 1973). Applying this experimental method we found that, at a given concentration of ADPRT and two of its polypeptides produced by digestion with plasmin and of 2.5 mM-glutaraldehyde (see the legend to Table 1), dimerization was readily detectable with ADPRT and its basic polypeptides of 29 kDa and 36 kDa (Table 1), as determined by SDS/PAGE. Predictably, SDS or quenching of glutaraldehyde with hydrazine prevented dimerization (Table 1). The percentage of dimer formation of ADPRT was a function of protein concentration (Fig. 5). However, the degree of dimerization of ADPRT as determined in this system cannot be generalized to conditions where self-association is studied in the absence of glutaraldehyde. Separation of monomeric ADPRT from the dimer produced by cross-linking with glutaraldehyde is illustrated also in Fig. 6, which shows an NEPHGE gel containing 6 M-urea (see the Experimental section). The identity of the dimer was confirmed by the doubling of its molecular mass as determined by SDS/PAGE (results not shown).

The association of polypeptides produced from ADPRT by digestion with plasmin was further determined with the aid of the

Table 1. Effect of experimental conditions on dimer formation of ADPRT and of two of its polypeptides by cross-linking with glutaraldehyde

ADPRT (18 μg) and its polypeptides obtained by digestion with plasmin (12 μg of 29 kDa polypeptide; 13 μg of 36 kDa polypeptide; mixture containing 9.5 μg each of 56 kDa and 42 kDa polypeptide) were treated with glutaraldehyde (2.5 mM) as described in the Experimental section, either in the absence or in the presence of SDS (50 mM). Polypeptide concentrations in the cross-linking system were adjusted to 0.3 mg/ml in all tests. Cross-linking was terminated by hydrazine sulphate (see the Experimental section), or hydrazine sulphate was added at zero time (Expt. 3). SDS/PAGE was performed with ADPRT in 6% acrylamide gels, and with the polypeptides in 10% acrylamide gels. Coomassie Blue-stained polypeptides were extracted and their concentrations determined as described in the Experimental section. Results represent duplicate analyses with an error limit of $\pm 15\%$.

Expt. no.	Experimental conditions of glutaraldehyde treatment	Proportion of dimer in the polypeptide solution (%)
1	ADPRT	16
2	ADPRT + SDS	0
3	ADPRT + quenching at zero time	0
4	29 kDa polypeptide	31
5	29 kDa polypeptide + SDS	0
6	36 kDa polypeptide	26
7	36 kDa polypeptide + SDS	0
8	56 kDa and 42 kDa polypeptides	0
9	56 kDa and 42 kDa polypeptides + SDS	0

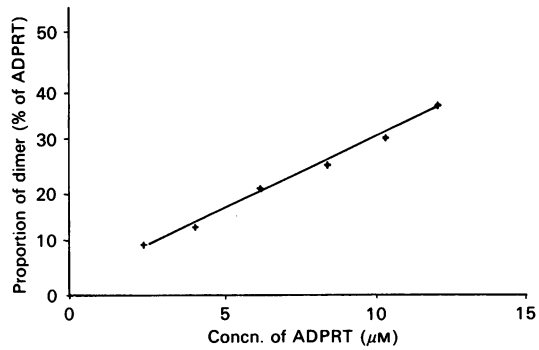

Fig. 5. Effect of the concentration of ADPRT on apparent dimer formation at pH 8.0, determined by cross-linking with glutaraldehyde and SDS/PAGE

ADPRT at various concentrations (see the abscissa) was treated with 2.5 mM-glutaraldehyde (see the Experimental section) and the dimer concentration was determined by SDS/PAGE followed by quantitative determination of Coomassie Blue-stained polypeptide bands (see the Experimental section). The concentration of ADPRT was plotted against the percentage proportion of ADPRT dimers.

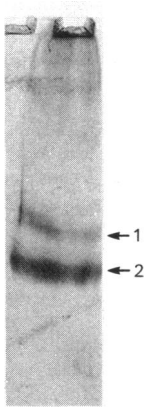

Fig. 6. Separation of the glutaraldehyde-cross-linked ADPRT dimer from monomeric ADPRT in NEPHGE gel

A 12 μg portion of ADPRT was cross-linked with glutaraldehyde (2.4 mM) in the presence of 5 μg of octameric duplex C for 10 min at 23 °C in a reaction volume of 20 μl. Samples from the hydrazine sulphate-quenched reaction mixture were loaded on to 6 M-urea-containing NEPHGE gel and electrophoresed as described in the Experimental section. Arrow 1 marks the position of the dimeric ADPRT in the electrophoretogram, and arrow 2 points to the monomer form of ADPRT.

reducible cross-linking reagent dimethyl 3,3′-dithiobispropionimidate, as illustrated in Fig. 7. The effect of time of incubation with the cross-linking agent on the electrophoretic behaviour of polypeptides was assayed without reduction of the disulphide (Fig. 7*a*) and following reduction (Fig. 7*b*). It is apparent that with progression of time the protein bands corresponding to 29 kDa and 36 kDa diminished, and large aggregates appeared on top of the gel when the disulphide cross-linker reacted with the polypeptides. A quantitative assessment is shown in Fig. 7(*c*), where the colorimetric readings (A_{595}) of extracted Coomassie Blue-stained polypeptides are plotted against time of incubation. It is apparent that the most significant cross-linking occurred with the 29 kDa and 36 kDa polypeptides, whereas the 56 kDa and 42 kDa polypeptides showed a much smaller tendency to self-associate. Reduction of the disulphide of the cross-linking agent re-established the polypeptide pattern obtained by gel electrophoresis (Fig. 7*b*).

Similar results were obtained by cross-linking with tetranitromethene, which is a far less efficient cross-linker than dimethyl 3,3′-dithiobispropionimidate. Only the 29 kDa and 36 kDa polypeptides exhibited trace amounts of dimer formation, as detected by silver staining, after incubation of the polypeptides with tetranitromethane. The same results were obtained when tetranitromethane-modified ADPRT was digested with plasmin (results not shown).

A comparison of the efficiencies of various cross-linking reagents was made by incubating 4.3 μM-ADPRT with 35 mM bifunctional cross-linkers for 20 min at pH 8.0 (100 mM-triethanolamine/HCl buffer) at 23 °C, followed by quantification of dimers by SDS/PAGE. Dimethyl pimelimidate and dimethyl suberimidate produced between 15.4 % and 21.4 % cross-linking, similar to that with glutaraldehyde (Table 1), and this cross-linking was uninfluenced by coenzymic octadeoxyribonucleotide duplex (1 μg/20 μl) or 250 mM-benzamide. However, dimethyl 3,3′-dithiobispropionimidate under the same conditions cross-linked 80.5–80.9 % of ADPRT, and this was depressed to 50 % by benzamide, but not by coenzymic deoxyribonucleotide duplex, suggesting a probable conformational alteration of ADPRT by benzamide that could alter accessibility of groups that react with the cross-linker.

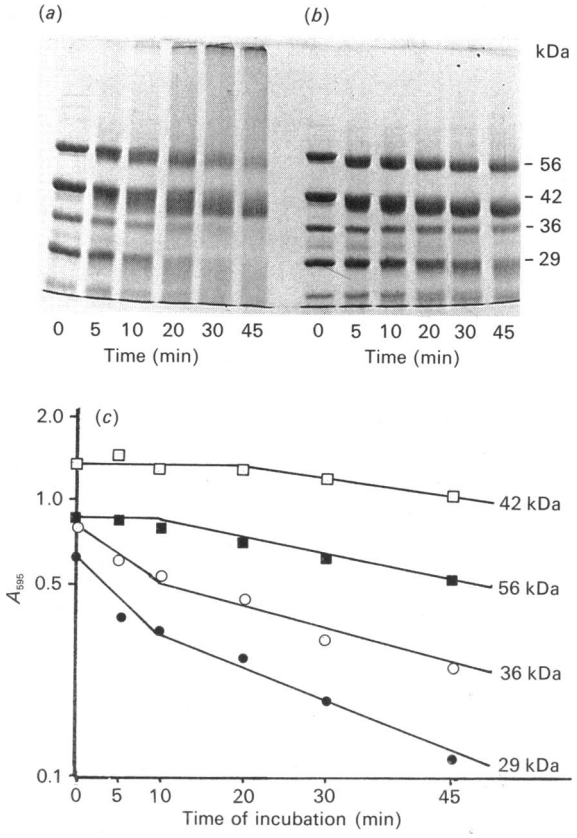

Fig. 7. Time-dependence of cross-linking with dimethyl 3,3′-dithiobispropionimidate of polypeptides of ADPRT obtained by digestion with plasmin

To a solution of 150 μg of polypeptides, made up in 75 μl of 100 mM-triethanolamine/HCl buffer, pH 8.0, was added an equal volume of 40 mM-dimethyl 3,3′-dithiobispropionimidate (dissolved in the buffer), and the mixture was incubated at 23 °C. At timed intervals 20 μl samples were withdrawn and quenched with 10 μl of 0.5 M-hydroxylamine solution, adjusted to pH 7.6. Two parallel samples (13 μl each) were then subjected to SDS/PAGE either without (*a*) or after (*b*) the reduction of the disulphide of the cross-linker. This was accomplished by mixing the samples with an equal volume of Laemmli sample buffer either containing 1 % (v/v) 2-mercaptoethanol or not. Gel electrophoresis and the quantification of the Coomassie Blue adsorbed on the polypeptides was carried out as described in the Experimental section. Panels (*a*) and (*b*) show the gel-electrophoretic separation of the polypeptides. The lower section (*c*) illustrates the quantities of polypeptides separated in (*a*).

Influence of self-association of ADPRT on enzymic activity and protein fluorescence

Results obtained by cross-linking appear to be consistent with a significant self-associative behaviour of ADPRT, and, since the polypeptides of 29 kDa and 36 kDa significantly aggregate by themselves, it is probable that the basic polypeptide components of ADPRT represent sites of self-association in the intact enzyme molecule. Since the polypeptide of 36 kDa represents one of the DNA-binding domains of the ADPRT protein (Buki & Kun, 1988; Sastry *et al.*, 1989), we assumed that self-association at these sites may diminish the rates of poly(ADP-ribose) synthesis from NAD^+ because self-association could interfere with the binding of DNA to ADPRT, which is a requirement for the coenzymic function of DNA in poly(ADP-ribose) synthesis. As illustrated in Table 2, the specific enzymic activity of ADPRT indeed exhibited marked dependence on the concentration of the enzyme protein present in the assay system, and a roughly 50-

fold dilution (to 20 nM-ADPRT concentration) maximized poly(ADP-ribose) polymerase activity.

Coincidental with the dilution of the enzyme protein from 2000 nM to 40 nM, there was a red-shift in protein fluorescence, suggesting protein dissociation (Lakowicz, 1983) that linearly correlated with the increase in specific enzymic activity (Fig. 8).

If dilution of the enzyme protein results in a progressive dissociation of aggregated molecules to maximally active dimers, and further dilution progressively to monomers, then results shown in Table 2 and Fig. 8 predict that the dimeric species of ADPRT should represent the maximally active form of the enzyme. This assumption was directly tested by assaying the specific activity of serially diluted ADPRT that was cross-linked by glutaraldehyde (see the legend to Fig. 9). In a typical experiment the enzymic activity of the glutaraldehyde-treated ADPRT was determined as 23.5 pmol of ADP-ribose/min per pmol of enzyme at 320 nM enzyme concentration, which corresponded to the interpolated specific activity given in Table 2 between dilutions 3 and 4, taking into consideration that cross-linking in the presence of the synthetic double-stranded octadeoxyribonucleotide coenzyme (Hakam *et al.*, 1987) preserved 70% of enzymic activity. We ascertained in separate experiments that glutaraldehyde did not cross-link between ADPRT and the octameric DNA analogue (results not shown). Dilution of the native enzyme, and to a small extent even of the glutaraldehyde-treated enzyme, produced the observed increase in specific activity (compare with Table 2), indicating that a fraction of ADPRT molecules in the glutaraldehyde-treated samples escaped cross-linking (results not shown). However, the decay of the specific activity that in the native enzyme occurred after further dilution to 40 nM enzyme did not take place with the glutaraldehyde-treated enzyme, consistent with a stabilization of covalently cross-linked dimers. This is illustrated in Fig. 9, where the dilution-dependent decrease in specific activity of the native enzyme was exponential with respect to the concentration of protein (curve *B*), whereas cross-linked dimers escaped

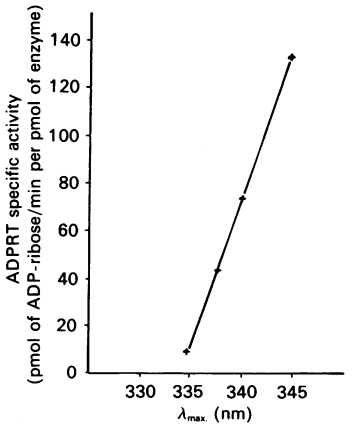

Fig. 8. Correlation between the concentration-dependent specific activity of ADPRT and the concomitant fluorescence emission maximum of ADPRT

The fluorescence spectra of ADPRT protein at various concentrations (800, 200, 160 and 40 nM) were recorded as described in the Experimental section. The emission maxima (abscissa) were plotted against the specific activities of ADPRT (ordinate), determined under identical conditions.

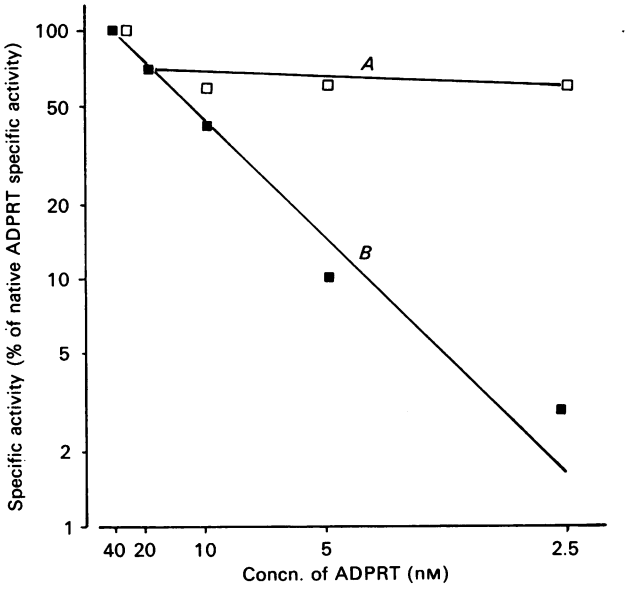

Fig. 9. Effect of dilution on the specific activity of native and glutaraldehyde-cross-linked ADPRT

Curve *A* (□): 12 μg of ADPRT was cross-linked with 2.5 mM-glutaraldehyde in the presence of 250 μg of octadeoxyribonucleotide duplex/ml for 10 min at 25 °C as described in the Experimental section. Curve *B* (■): same procedure, except that the quenching agent hydrazine sulphate was added at zero time. The specific enzyme activities of samples from serial dilutions (ordinate) were plotted against the respective enzyme concentrations (abscissa). The glutaraldehyde-treated ADPRT retained 70% of enzymic activity, determined at 320 nM-ADPRT concentration (for details see the text).

Table 2. Effect of the concentration of ADPRT on the specific activity of the enzyme

The specific activity of 2 μg of ADPRT was determined in an increasing reaction volume between 10 and 4000 μl, which gives the ADPRT molar concentration range shown in the first column. The two last ADPRT concentrations were obtained by first diluting the stock solution of ADPRT 10-fold, in order to decrease the total reaction volume. The enzymic assay system consisted of 100 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA, 304 nM (saturating concentration) double-stranded synthetic octadeoxyribonucleotide coenzyme (Hakam *et al.*, 1987) and 200 μM-[^{32}P]NAD$^+$ (10 c.p.m./nmol). The enzymic reaction was initiated by the addition of 1 μl of ADPRT to different volumes of the reaction mixture (see above). The assay was run at 25 °C and was terminated by 20% (w/v) trichloroacetic acid (final concn.) within 1–5 min, which corresponds to enzymic rates linear with time. [^{32}P]ADP-ribose covalently bound to ADPRT was quantified by radiochemical assay (Hakam *et al.*, 1987). Data are calculated from four series of analyses, where the S.D. values of the specific activities vary in the range ±5–10% of the averages listed.

Concn. of ADPRT (nM)	ADPRT specific activity (pmol of ADP-ribose/min per pmol of enzyme)
2000	4.55
800	9.2
400	18.6
200	44.6
160	74.2
80	115.2
40	133.2
20	166.7
8	143.5
4	44.4
3	8.1
2	4.2
1	5.2

Table 3. Enzymic activities *in vitro* of monomers and dimers of ADPRT separated in NEPHGE gels

A 12 μg portion of ADPRT was treated with glutaraldehyde (2.5 mM) in the presence of 250 μg of octadeoxyribonucleotide duplex C/ml for 10 min at 23 °C (see the Experimental section). In control experiments (representing native enzyme) hydrazine sulphate was added to the incubation mixture before the addition of the cross-linking agent. After addition of Ampholine (pH 1–3) to 1 % final concentration the samples were loaded on to the NEPHGE gels. After electrophoresis (see the Experimental section) the gels were incubated first with 0.1 M-Tris/HCl buffer, pH 8.0, followed by a 30 min incubation in 10 ml of assay mixture containing no NAD^+. Finally, [^{32}P]NAD^+ (50 μCi; 1 μM final concentration) was added and the incubation was continued for 30 min at room temperature. The remaining NAD^+ was washed out by subsequent incubations in excess volumes of 20 % (w/v) trichloroacetic acid. The gel was then sliced into 1 mm sections, and the radioactivity of each slice was determined by liquid-scintillation spectrometry. The amount and the location of monomeric and dimeric ADPRT were determined as described in the Experimental section. The results are given as means ± S.D. for three parallel experiments.

	Non-cross-linked		Cross-linked	
	Amount of protein (pmol)	ADPRT specific activity (pmol of ADP-ribose/min per pmol of enzyme)	Amount of protein (pmol)	ADPRT specific activity (pmol of ADP-ribose/min per pmol of enzyme)
ADPRT monomer	83.3 ± 2.7	21.9 ± 3.8	50.0 ± 5.2	57 ± 14.4
ADPRT dimer	0.4 ± 0.25	76.8 ± 9.6	33.3 ± 2.8	169.3 ± 14

the dilution-dependent decay of specific polymerase activity, indicating that at this dilution the enzymic rate was limited by cross-linked dimers.

A direct comparison of enzymic activities of enzyme solutions containing monomeric and dimeric ADPRT molecules was carried out after their separation in NEPHGE gels with both native and glutaraldehyde-cross-linked enzymes. As illustrated in Table 3 there is a significant increase of the specific activity of dimers, and it is probable that the relatively low specific activity of monomers may be due to contamination by dimers or to self-association of monomers as a consequence of the high concentration of ADPRT in the gel. Monomers and dimers in the NEPHGE gel were identified by molecular markers, consisting of native ADPRT and glutaraldehyde-cross-linked ADPRT, that gave a doubling of molecular mass in SDS/PAGE, and both species were also identified by immunotransblots. Although some degree of cross-contamination of monomers by dimers cannot be avoided, the marked increase in the specific enzymic activities of cross-linked dimers is consistent with the interpretation that maximal catalytic activity correlates with dimerization.

Base hydrolysis, above pH 6.5, of an unstable ADP-ribose–enzyme initiator adduct accounts for the known NAD^+ glycohydrolase activity of ADPRT (Bauer *et al.*, 1986). Apparent NAD^+ glycohydrolase activity thus necessarily accompanies auto-poly(ADP-ribosyl)ation of ADPRT when enzymic reactions are performed at pH 8.0. When polymerase and NAD^+ glycohydrolase activities were assayed simultaneously by determining the free ADP-ribose formed from NAD^+ in a system that also synthesized ADP-ribose–polymer adducts, and both enzymic activities were compared at varying ADPRT concentrations, the results summarized in Table 4 were obtained. The steadily increasing free ADP-ribose is in marked contrast with the quantity of protein-bound homopolymers, which followed the pattern shown in Table 2. It follows that at high enzyme dilution, which tends to dissociate ADPRT into monomers, NAD^+ glycohydrolase activity is sustained, even greatly increased, predicting that the monomeric ADPRT is an NAD^+ glycohydrolase.

Table 4. Effect of ADPRT concentration on the formation of free ADP-ribose and enzyme-bound polymer from NAD^+

The experimental conditions were the same as described in the legend to Table 2. Free ADP-ribose was assayed by quantitative h.p.l.c. (Hakam & Kun, 1985). Each value represents the average for two experiments with error limits.

Concn. of ADPRT (nM)	Product formed (pmol/pmol of ADPRT)	
	Free ADP-ribose	Poly(ADP-ribose)
800	5.0 ± 0.2	10.5 ± 2.3
200	10.7 ± 2.1	61.1 ± 3.2
20	33.0 ± 0.4	128.4 ± 0.6
2	66.3 ± 16.1	12.6 ± 3.8

DISCUSSION

The regulation of the metabolic enzymic activity of ADPRT, which is the process of poly(ADP-ribose) formation from NAD^+, has been predominantly related to the formation and accessibility of DNA strand breaks. In fact, only trace polymerase activity can be detected in intact cells by adenine labelling, as reported by Aboul-Ela *et al.* (1988), and extensive DNA damage by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine had to be applied to obtain significant enzymic rates (Aboul-Ela *et al.*, 1988; Rankin *et al.*, 1989). However, the enzyme content of a large variety of eukaryotic cells is surprisingly high (Yamanaka *et al.*, 1988; Ludwig *et al.*, 1988), and it has been calculated that under physiological conditions only 1 % of the enzyme protein accounts for poly(ADP-ribosyl)ation. Janssen & Hilz (1989) concluded that hardly any correlation exists between certain cell biological phenomena, such as differentiation of 3T3-L1 cells, and rates of poly(ADP-ribosyl)ation as tested by inhibitors. The reported topological effect of ADPRT exerted on DNA conformation was inhibited by NAD^+, and this inhibition was counteracted by ADPRT ligands that need not be structural analogues of NAD^+ (Hakam *et al.*, 1987; Tseng *et al.*, 1987; Sastry & Kun, 1988; Sastry *et al.*, 1989). This binary mechanism may explain a physiological function of ADPRT that consists of an induction of DNA conformational change following ADPRT ligand-induced DNA binding of ADPRT. However, the low rate of poly(ADP-ribosyl)ation that occurs under physiological conditions and may be correlated to DNA replication may have additional and possibly important self-regulatory functions. For example, the ADP-ribosylation of histone H3 in stimulated cells (Sooki-Toth *et al.*, 1989) may be a specific de-repressing signal to the genome by counteracting the known repressor role of the histone. The second known enzymic function of ADPRT, which is NAD^+ glycohydrolase activity (cf. Althaus & Richter, 1987), has not been so far considered in terms of cellular physiology.

Owing to limitations of experimental technologies some of the methods used for the determination of macromolecular association of ADPRT would not be convincing if applied alone. Molecular filtration by itself contains ambiguities by possibly signalling anomalous molecular symmetries (Regnier, 1983), and cross-linking, being dependent on the concentration of the reaction components, indicates equilibria, and changes in fluorescence due to dissociation of oligomeric proteins are indirect. However, cross-linking reagents can identify molecular contacts between macromolecules. At a constant concentration of cross-linking reagents the detection of macromolecular association of ADPRT and its polypeptides obtained by plasmin digestion depends also on the rates of reactions of cross-linking agents with macromolecules. Cross-linkers with relatively low reaction rates (approx. 20% cross-linking during 20 min) detect only the most easily occurring associations (e.g. between the basic polypeptides), whereas dimethyl 3,3′-dithiobispropionimidate, which under the same conditions cross-links approx. 80% of macromolecules, is able to detect some degree of association of polypeptides other than the basic ones, although the latter are still predominating. However, these limitations imposed by the differences in the chemical reactivity of cross-linkers do not invalidate the conclusion that the most probable sites of self-association of ADPRT are its basic polypeptide domains.

Application of non-denaturing gel techniques in combination with enzymic assays *in situ* in gels where ADPRT has been separated into the monomeric and dimeric forms constitute a more direct identification of differences in specific enzymic activities that can be correlated with molecular aggregation. Regardless of what technique was used, results converged on the conclusion that poly(ADP-ribose) polymerase activity is markedly dependent on self-association of ADPRT, being maximal when ADPRT appears to be dimeric, and activity is significantly depressed by higher order of self-association or by dissociation to monomers by further dilution. Since the basic polypeptide components of ADPRT by themselves self-associate, it is reasonable to assume that ADPRT molecules bind to each other at the basic polypeptide domains, thus competing for the binding of the coenzymically required DNA (Buki & Kun, 1988), which partly explains the low specific activity at high protein concentration. Assuming a nuclear volume of 148 μm^3 (Mellors *et al.*, 1953), the ADPRT content of nuclei is close to 1 μM, which coincides with the inhibitory enzyme concentrations found *in vitro* (Table 2). More extensive dilution, which predictably tends to dissociate dimers to monomers, also dramatically diminishes poly(ADP-ribose) polymerase activity, suggesting that effective poly(ADP-ribosyl)ation of ADPRT molecules requires two associated enzyme molecules, one presumably being the initiator catalyst and the second the ADP-ribose acceptor, a possibility that has been proposed previously (Bauer & Kun, 1985; Bauer *et al.*, 1986; Kameshita *et al.*, 1986). It seems plausible that both molecules may simultaneously act as both initiator catalysts and also acceptors, which explains the unsuitability of kinetics alone to discriminate between mono- and bi-molecular catalysis. The surprising emergence of a large increase in NAD^+ glycohydrolase activity that follows dilution may have two explanations. Dilution may partially denature ADPRT, which could induce artifactual NAD^+ glycohydrolase activity, or alternatively the monomeric ADPRT may be a genuine DNA-dependent NAD^+ glycohydrolase, operative in NAD^+ catabolism *in vivo*. As we find, histones activate both poly(ADP-ribose) synthetase and NAD^+ glycohydrolase activities, the latter even at high dilutions (P. I. Bauer, A. Hakam & E. Kun, unpublished work); a protein-dilution-induced denaturation seems unlikely. For these reasons a physiologically functional NAD^+ glycohydrolase activity of ADPRT cannot be ruled out. Results shown in this paper constitute model experiments *in vitro* that may represent a molecular control of ADPRT *in vivo* by mechanisms that cannot be recognized in complex cellular systems but can be shown only in reductionist models *in vitro*. Notably the mechanism and cell-regulatory role of many DNA-specific enzymes, such as DNA polymerases, topoisomerases, helicases etc., cannot be determined in the intact cell, but can be shown only in isolated systems, and their cellular function can be recognized by inhibitors only. Thus a proposed cellular regulation by ADPRT *in vivo* as tested with ADPRT ligands is not without precedents.

The macromolecular self-association of ADPRT contributes an additional regulatory mechanism of the metabolic, i.e. poly(ADP-ribose) synthesizing and NAD^+-hydrolysing, functions of this enzyme.

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