Free ADP-ribose in human erythrocytes: pathways of intra-erythrocytic conversion and non-enzymic binding to membrane proteins

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We have previously identified free ADP-ribose (ADPR) as a normal metabolite in mature human erythrocytes. In this study the metabolic transformations of ADPR were investigated in both supernatants from erythrocyte lysates and intact erythrocytes, loaded with ADPR by means of a procedure involving hypotonic haemolysis and isotonic resealing. In both experimental systems, the main pathway was a dinucleotide pyrophosphatase-catalysed hydrolysis to yield AMP, which was readily converted into the adenylic and inosinic nucleotide pools. To a lesser extent, ADPR underwent conversion into a compound that was identified as ADP-ribulose (ADPRu), on the basis of m.s., n.m.r. spectroscopy and enzymic analysis. ADPRu was also susceptible to degradation by the dinucleotide pyrophosphatase, which was partially purified from erythrocyte lysates and charac-

terized with respect to its substrate specificity. Isomerization of ADPR to ADPRu was markedly enhanced by ATP. Incubation of unsealed haemoglobin-free erythrocyte membranes with labelled ADPR did not cause any transformation of this nucleotide and resulted in its trichloroacetic acid- and formic acid-resistant binding to a number of membrane cytoskeletal proteins. These proteins include spectrin, glyceraldehyde 3-phosphate dehydrogenase (Ga3PDH), three proteins of molecular masses 98, 79 and 72 kDa, which apparently comigrate with bands 3, 4.1 and 4.2 respectively, and two additional proteins of molecular masses 58 and 41 kDa. Acid-resistant binding of ADPR, as well as of NAD⁺, to Ga3PDH was confirmed for the enzyme purified from human erythrocytes.

INTRODUCTION

Until now, little information has been available on the content of free ADP-ribose (ADPR) and its metabolism in animal cells. Knowledge of the parameters might shed light on the physiological relevance of non-enzymic ADP-ribosylation, i.e. the transferase-independent covalent binding of free ADPR to specific acceptor proteins. By forming Schiff bases capable of conversion into stable products, free ADPR can modify lysine, arginine and/or histidine residues in proteins [1]. In addition, other chemical groups of proteins (e.g. thiol and carboxylate groups) can link ADPR [2]. Indeed, non-enzymic ADP-ribosylation has been described in several biological systems [2–8].

One of the most serious difficulties to overcome for the determination of the concentration of free ADPR *in vivo* is the degradation of nicotinamide nucleotides to ADPR during both the acid and the alkaline treatments that are conventionally used for the extraction of nucleotides from cell homogenates [2]. NADH and NAD⁺ are converted into ADPR during acid and alkaline treatment respectively [9,10]. Haemoglobin present in erythrocytes, however, can prevent the acid-induced hydrolysis of NADH and NADPH by promoting their complete oxidation to the corresponding acid-resistant oxidized forms [9,11]. In addition, unavailability of intra-erythrocytic NAD(H) to the phosphatidylinositol-anchored ectoenzyme [12–14], NAD⁺ glycohydrolase, prevents generation of ADPR throughout the extraction procedures [11]. Finally, the possible presence of free ADPR in human erythrocytes was suggested by the recent

purification from this source of the enzymes involved in the transfer of ADPR from donor NAD⁺ molecules [15] and its hydrolysis from ADP-ribosylated proteins [16]. These findings prompted us to investigate the presence of free ADPR in human erythrocytes. Indeed, free ADPR was detected in mature human erythrocytes at $0.45 \pm 0.1 \mu\text{M}$ and also found to be formed intracellularly [11].

The demonstration of free ADPR in the erythrocyte raises several questions on its turnover and its possible functional roles. In this paper we addressed these problems by investigating the pathways of ADPR transformation in haemolysates and in intact erythrocytes and also its non-enzymic binding to erythrocyte membrane proteins.

MATERIALS AND METHODS

Materials

All enzymes utilized were from Sigma Chemical Co., St. Louis, MO, U.S.A. [³²P]ADPR was prepared by incubation of 8×10^7 c.p.m. of [³²P]NAD⁺ (30 Ci/mmol; NEN, Florence, Italy) in 0.2 ml of PBS with 0.6 unit of NAD⁺ glycohydrolase (EC 3.2.2.5, from *Neurospora crassa*). After 90 min, the incubation was stopped and the mixture deproteinized with trichloroacetic acid (5% final concentration). Trichloroacetic acid was removed with three successive extractions with diethyl ether. Excess diethyl ether was evaporated under nitrogen and the [³²P]ADPR formed was separated from both nicotinamide and the unchanged [³²P]NAD⁺ by means of the phosphate h.p.l.c.

Abbreviations used: ADPR, ADP-ribose; ADPRu, ADP-ribulose; TEA, tetraethylammonium hydroxide; TPA, tetrapropylammonium hydroxide; DSS, 4,4'-dimethyl-4-silapentane sodium sulphonate; TSP, trimethylethylsilyl sodium propionate; COSY, correlation spectroscopy; Ga3PDH, glyceraldehyde 3-phosphate dehydrogenase.

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described below. The peak corresponding to ADPR was collected, desalted by formate h.p.l.c. (see below) and stored at -20°C .

[*carboxyl*- ^{14}C]NAD $^{+}$ (41 mCi/mmol; Amersham, Milan, Italy) and [*adenylate*- ^{32}P]NAD $^{+}$ (30 Ci/mmol, NEN) were re-purified by h.p.l.c. (see below) before use. Cyclic ADPR was kindly provided by Dr. H. C. Lee, University of Minnesota, Minneapolis, MN, U.S.A.

Spectrin, purified from human erythrocytes, was obtained from Sigma. Ga3PDH was purified from human erythrocyte membranes as described by Rogalski et al. [17].

Preparation of haemolysates

Blood samples were drawn from normal volunteers after informed consent. Heparin was used as anticoagulant, and leucocytes were removed with a leucocyte removal filter (Sepacell, Asahi Medical Co. Ltd., Tokyo, Japan). Experiments with defibrinated blood samples gave strictly comparable results, thus excluding platelets as a source of appreciable amounts of ADPR-converting enzymes. Haemolysates were prepared by dilution of washed packed erythrocytes in ice-cold water to a final haematocrit of 30% and subsequent centrifugation at 16000 *g* for 20 min at 4°C to remove membranes. The top 10% layer of the supernatants, showing no detectable acetylcholinesterase activity as a marker of erythrocyte membranes [18], was then used for all experiments.

Preparation of erythrocyte membranes

Washed packed erythrocytes were haemolysed in 10 vol. of ice-cold 5 mM phosphate buffer, pH 8.0, and centrifuged at 16000 *g* for 20 min at 4°C . Erythrocyte ghosts were further washed four times in 10 vol. of the same buffer. The white, packed membranes (5–7 mg/ml protein concentration) were stored at -20°C .

Labelling of membrane-bound proteins with [^{32}P]ADPR

After incubation of 450 μg of ghosts in 5 mM phosphate buffer, pH 8.0, with 2 mM MgCl_2 and 3×10^7 c.p.m. of [^{32}P]ADPR (6×10^7 c.p.m./nmol) for 60 min at 37°C , the non-covalently bound nucleotide was removed by acid extraction [19]. After addition of trichloroacetic acid (15% final concentration) and centrifugation, the acid-insoluble material was washed three times with 1 ml of 5% trichloroacetic acid and dissolved in 0.1 ml of ice-cold 98% formic acid. The sample was then diluted with 0.9 ml of water and the proteins were precipitated with 200 mg of trichloroacetic acid. The pellet was again washed three times with 1 ml of 5% trichloroacetic acid and redissolved in PAGE buffer. The sample was heated at 100°C for 5 min, divided into two portions and subjected to SDS/PAGE (10% acrylamide), as described by Laemmli [20]. The gel was cut longitudinally and one lane was stained with Coomassie Blue, dried and exposed to a Hyperfilm-MP (Amersham) film for 7–10 days at -20°C . The other excised lane was homogenized in 7 ml of 5 mM Tris/HCl, pH 8.0, with 0.01% SDS, and the proteins were electroeluted for 3 h in the same buffer. After removal of the polyacrylamide by centrifugation, the supernatant was dialysed overnight against 5 litres of 5 mM Tris/HCl, pH 8.0. The dialysed supernatant was dried in a rotary evaporator, redissolved in 600 μl of water and divided into two portions: one was incubated with 6 μg of snake

venom pyrophosphatase (EC 3.6.1.9, from *Crotalus adamanteus*) in the presence of 5 mM MgCl_2 . The other one was incubated under the same conditions without the enzyme. After 60 min at 37°C the incubations were stopped with trichloroacetic acid: the precipitates were washed twice in 5% trichloroacetic acid and counted, and the supernatants were injected into the phosphate h.p.l.c. system (see below). Fractions were collected every 0.5 min and counted for radioactivity.

H.p.l.c. analyses

H.p.l.c. analyses were performed on a Hewlett–Packard HP1090 instrument equipped with an HP 1040 A diode array spectrophotometric detector set at 260 nm. The column was a 5 μm , 100 mm $\times$ 2.1 mm, ODS Hypersil C $_{18}$ (Hewlett–Packard, Milan, Italy) and the flow rate was 0.5 ml/min. Two different analyses were used.

Phosphate analysis

Solvent A was 0.1 M KH_2PO_4 containing 5 mM PicA reagent (Millipore, Milan, Italy) with the pH adjusted to 6.5 with 5 M KOH; solvent B was 70% A and 30% methanol, with the pH adjusted to 7.5 with 5 M KOH. The solvent program was a gradient starting at 100% solvent A for 5 min, linearly increasing to 2% solvent B in 3 min, then linearly increasing to 50% solvent B in 24 min. The column was re-equilibrated in solvent A for 10 min before the next injection. The elution times (in minutes) for the various compounds were the following: free phosphate and ribulose 5-phosphate, 0.8; inosine, 0.9; nicotinamide, 1.2; IMP, 1.6; NAD $^{+}$, 2.0; IDP-ribose, 2.8; AMP, 3.2; ADPR, 7.0; adenylic metabolite of ADPR (ADPr), 8.0; ADP, 9.0; ATP, 18.0.

Formate analysis

Solvent A was 0.01 M formic acid with the pH adjusted to 4.0 with 1.3 M TEA. Solvent B was 50% solvent A and 50% methanol. The solvent program was a gradient starting at 100% solvent A for 5 min, then linearly increasing to 100% solvent B in 10 min. ADPR and ADPr were co-eluted at 12 min and were separated from free phosphate (0.8 min). Integration of peak areas were obtained by means of a Hewlett–Packard 79996A analytical workstation and quantification of results was achieved by relating integrated peak areas to peaks obtained with high-purity standard compounds run separately.

Purification of the adenylic metabolite of ADPR

Haemolysates were incubated with 1 mM ADPR and 1 mM ATP at 37°C . After 60 min the trichloroacetic acid extract was subjected to phosphate h.p.l.c. and the peaks corresponding to the adenylic metabolite and untransformed ADPR were collected, dried, redissolved in water and subjected separately to formate h.p.l.c. The phosphate-free peaks were collected, dried and redissolved in either distilled water for m.s. and n.m.r. spectroscopy or PBS for incubation with phosphoriboisomerase (EC 5.3.1.6, from *Torula* yeast).

H.p.l.c./m.s. analysis

Mass spectra were acquired using a triple quadrupole tandem mass spectrometer PE-Sciex API III equipped with an ionspray atmospheric pressure ionization source. The mobile phase flow was introduced, without splitting, to the source. Mass spectra

were acquired in negative mode scanning over a range that included the expected molecular masses of ADPR and related compounds as well as possible adducts with the ion-pair reagent used for the chromatography.

Separations were performed with an Applied Biosystem 140A syringe pumps h.p.l.c. system and an autosampler Perkin-Elmer ISS-101. U.v. detection was performed at 260 nm with an Applied Biosystem diode array detector 1000S and computed through an analogic channel of the mass spectrometer. A reversed-phase column (Hypersil BDS 5 μ m, 25 mm $\times$ 1 mm column from Shandon) was used with a mobile phase composed of 3.3 mM tetrapropylammonium hydroxide (TPA) brought to pH 4.0 with formic acid in water (solvent A) and 3.3 mM TPA brought to pH 4.0 with formic acid in acetonitrile/water (9:1, v/v) (solvent B). The elution conditions adopted consisted of 3 min of isocratic 100% solvent A, followed by a linear gradient to 50% of solvent B in 20 min and by a final isocratic step of 7 min at the last composition. The flow rate was 50 μ l/min. Under these conditions, the elution times of ADPR and its adenylic metabolite were 24.1 and 25.8 min respectively.

N.m.r. spectra

^1H -n.m.r. spectra were recorded at 500 MHz with a Bruker AMX 500 spectrometer equipped with a 5 mm $^1\text{H}/\text{X}$ inverse probehead. The adenylic compound formed from ADPR in erythrocyte lysates (500 μ g) was solubilized in $^2\text{H}_2\text{O}$ (0.5 ml, 99.99 $^2\text{H}\%$). Spectra were recorded at 25 $^\circ\text{C}$ and 30 $^\circ\text{C}$, to shift residual water signal. Commercial ADPR and ribulose 5'-phosphate, both from Sigma, were used as reference compounds. All proton shifts were referred to internal 4,4'-dimethyl 4-silapentane sodium sulphonate (DSS). All carbon shifts were referred to external trimethylethylsilyl sodium propionate (TSP).

Encapsulation of [^{32}P]ADPR or [^{32}P]NAD $^+$ within erythrocytes

Encapsulation was obtained by transient hypotonic lysis, after pre-swelling of the washed erythrocytes, followed by isotonic resealing. Briefly, erythrocytes were incubated in 10 vol. of 200 mOsm KCl for 5 min at 25 $^\circ\text{C}$ and then centrifuged at 1100 g for 5 min. Packed erythrocytes (haematocrit 80%) were then haemolysed by addition of 0.3 vol. of aq. 2 mM [^{32}P]NAD $^+$ (specific radioactivity 12000 c.p.m./nmol) or [^{32}P]ADPR (same specific radioactivity). After 10 min at 25 $^\circ\text{C}$, the opened erythrocytes were resealed by addition of 0.1 vol. of resealing solution [21], incubated for 10 min at 37 $^\circ\text{C}$ and washed four times in 10 vol. of 0.15 M NaCl before being submitted to experiments designed to follow the intracellular metabolism of these nucleotides.

Purification of ADPR pyrophosphatase

The enzyme was partially purified from haemolysates by a two-step chromatography on DEAE-cellulose and NAD $^+$ -agarose (Sigma). Briefly, the haemolysate (30 ml) was applied to a DEAE-cellulose column (8 ml bed volume) equilibrated in 10 mM Tris/HCl, pH 8.3, containing 0.5 mM EDTA (Tris/EDTA). After the column had been washed with 200 mM KCl in Tris/EDTA, the haemoglobin-free pyrophosphatase activity was eluted with 300 mM KCl in Tris/EDTA. After overnight dialysis against Tris/EDTA, the DEAE eluate was mixed with 3 ml of NAD $^+$ -agarose pre-equilibrated in Tris/EDTA in a conical centrifuge tube. After 30 min at 0 $^\circ\text{C}$ under constant mixing, the resin was thoroughly washed by centrifugation at 600 g and the bound activity was released using three successive elutions with

1 vol. of 50 mM KCl in Tris/EDTA each. The eluate was dialysed against 10 mM Tris/HCl, pH 8.3, and stored at -20°C .

ADPR pyrophosphatase activity was assayed in 10 mM Tris/HCl, pH 8.3, with 1 mM substrate and 10 mM MgCl_2 . At various incubation times at 37 $^\circ\text{C}$, samples were removed, deproteinized with trichloroacetic acid (5% final concentration), centrifuged and the excess trichloroacetic acid removed with diethyl ether. Samples were subjected to phosphate h.p.l.c.

Protein concentration was determined by the method of Bradford [22].

RESULTS

Formation of an adenylic compound from ADPR incubated in haemolysates

The metabolic fate of ADPR in human erythrocytes was first investigated in haemolysates. When membrane-free haemolysates were incubated for 60 min at 37 $^\circ\text{C}$ with [^{32}P]ADPR, h.p.l.c. analysis of the corresponding trichloroacetic acid extracts demonstrated a marked consumption of this labelled compound (Figure 1b). At the same time, a large amount of AMP was produced together with a small amount of a radioactive h.p.l.c. peak, with an absorption spectrum typical of adenine nucleotides, well separated from adenosine, AMP, ADPR, ADP and ATP. Assuming the A_{260} absorption coefficient to be similar to that of ADPR and ATP, the specific radioactivity of the adenylic compound was 900 c.p.m./nmol, i.e. comparable with that of ADPR and remarkably higher than that of ATP (50 c.p.m./nmol). Addition of 1 mM ATP to the haemolysate increased the amount of newly formed adenylic compound and decreased ADPR consumption considerably (Figure 1c).

The adenylic metabolite was completely unlabelled when incubation of the haemolysate was carried out with unlabelled ADP and radioactive [^{32}P]ATP (either [α - ^{32}P]ATP or [γ - ^{32}P]ATP, not shown). Thus the newly formed adenylic compound is synthesized from ADPR as a precursor and ATP promotes its formation either by stimulating synthesis or through inhibition of degradation.

Identification of the adenylic compound formed from ADPR

After incubation of the haemolysate with unlabelled ADPR in the presence of ATP (under the same conditions as in the experiment of Figure 1), both the residual ADPR and the newly formed adenylic metabolite were purified by h.p.l.c. Both fractionated compounds were subjected to m.s. and n.m.r. spectroscopy.

Under the chromatographic conditions adopted for the h.p.l.c./m.s. analysis (see the Materials and methods section), the retention times of ADPR and its adenylic metabolite were 24.1 and 25.8 min respectively. Figures 2(a) and 2(b) show the mass spectra obtained from these peaks. In the spectrum of the peak eluted at 24.1 min (Figure 2a), as expected, the most intense ion, m/z 558, had the m/z value of a singly charged deprotonated molecular ion ($M-H$) $^-$ of ADPR. Another relevant ion was also present, m/z 743, corresponding to an adduct with the ion-pair reagent ($M+TPA-2H$) $^-$; in fact the mass difference, with respect to the ($M-H$) $^-$, was 185 Da (the molecular mass of TPA is 186 Da). In the spectrum of the unidentified adenylic peak eluted at 25.8 min (Figure 2b) the m/z 558 ion was also present and intense, but two other ions of relevant intensity, m/z 526 and 499, could also be observed. As in the previous spectrum, with m/z shifted by 185 Da, there were ions corresponding to TPA adducts of all the above-mentioned ions (743, 711, 684). The patterns of fragmentation of the unknown metabolite are con-

Figure 1 Incubation of haemolysate with ADPR

The haemolysate was incubated with 1 mM ADPR and 10^7 c.p.m. of [32 P]ADPR with or without 1 mM ATP. At 0 and 60 min, 1 vol. of the incubation mixture was deproteinized with 1 vol. of 10% trichloroacetic acid as described in the Materials and methods section. Trichloroacetic acid extracts were injected into the phosphate h.p.l.c. system and fractions collected every 30 s for determination of radioactivity. —, Chromatography profile; hatched area, radioactivity. (a) zero time; (b) 60 min without ATP; (c) 60 min with ATP.

sistent with loss of a $-\text{CH}_2\text{OH}$, with molecular rearrangement and further loss of a proton (m/z 526 in Figure 2b) and with loss of a $\text{CH}_2\text{OH}-\text{CO}$ (m/z 499 in Figure 2b) respectively. As ADPR and the unknown adenylic metabolite exhibit the same m/z for the relevant molecular ions, they must be isomeric compounds.

Figure 2(c) shows the reconstructed chromatographic traces at m/z values of 558, 526 and 499. These data confirm that both chromatographic peaks (24.1 and 25.8 min) contained ions with m/z values corresponding to deprotonated molecular ions from ADPR, whereas the ions with m/z lower than $(M-\text{H})^-$ of ADPR could be observed only in the peak of its unknown derivative. This fragmentation presumably involves a terminal part of the molecule, as the fragments showed little reduction in molecular mass.

To summarize, the m.s. results indicate that the unidentified adenylic metabolite has the same molecular mass as ADPR, but a different structure, which is more susceptible to fragmentation than ADPR itself. Isomerization of ribose into ribulose is compatible with the patterns of fragmentation observed in the metabolite.

The n.m.r. spectra of ADPR and its adenylic metabolite (both purified from erythrocyte cytosol as described above) are illustrated in Figure 3. The proton spectra of ADPR (Figure 3a) show one resonance, involving the glycosidic bond, in the anomeric region (6.19 p.p.m.). Through homonuclear correlation spectroscopy (COSY) analysis (not shown), it was possible to identify all other protons of the ribose ring (H2 4.79 p.p.m.; H3 4.50 p.p.m.; H4 4.38 p.p.m.; H5 4.13 p.p.m.). These resonances correspond to the non-reducing ribose moiety of standard ADPR, to which we had previously assigned all signals. The peaks at 5.35 and 5.21 p.p.m. correspond to the reducing protons of the terminal (i.e. reducing) ribose moiety of ADPR.

In the spectrum of the adenylic metabolite under study (Figure

3B) the peaks at 5.35 p.p.m. and 5.21 p.p.m. disappeared. The other signals attributable to the ribose residue (H1 6.22 p.p.m.; H2 4.79 p.p.m.; H3 4.53 p.p.m.; H4 4.41 p.p.m.; H5 4.15 p.p.m.) were somewhat displaced compared with those of the non-reducing ribose of ADPR. Non-ribose signals (at 4.40, 4.10 and 3.96 p.p.m.) were present in the spectrum, but by homonuclear COSY (not shown) it was possible to determine their correlations, while a CH_2 signal at 4.57 p.p.m. showed no correlation with the other ones. A similar correlation pattern was found for standard ribulose 5'-phosphate. In particular, signals at 3.8–3.9 p.p.m. were assigned by heteronuclear $^1\text{H}-^{31}\text{P}$ decoupling to protons of carbon 5 (R'5), whereas *geminal* protons of carbon 1 (R'1) gave a complex pattern of signals at 4.5–4.65 p.p.m.. By means of $^1\text{H}-^{13}\text{C}$ inverse COSY of the adenylic metabolite (Figure 3c), it was possible to assign all ^{13}C chemical shifts. The peaks at 4.40 p.p.m. and 4.10 p.p.m. (corresponding to carbon signals at 77 p.p.m. and 73 p.p.m. respectively) are secondary alcohols, and those at 4.57 p.p.m. (^{13}C : 69.5 p.p.m.) and 3.96 p.p.m. (^{13}C : 66.8 p.p.m.) correspond to primary alcohols. The low intensity of the proton signal at 3.96 p.p.m. in the crowded region of the spectrum (4.3–3.9 p.p.m.) suggests the presence of a signal corresponding to another geminally correlated proton.

In conclusion, the n.m.r. spectra of the adenylic metabolite of ADPR suggest the presence of an ADP residue linked to a $-\text{O}-\text{CH}_2-\text{CHOH}-\text{CHOH}$ moiety also containing a $-\text{CH}_2$ group bonded to a carbon bearing no protons. Together with the m.s. data, the n.m.r. spectra support the structure of ADPRu for the adenylic compound. This conclusion is also suggested by the overlap of the two-dimensional inverse heteronuclear correlation maps of the compound under study and of standard ribulose 5'-phosphate in the region of the ribulose signals (not shown).

Further experiments were carried out on the adenylic metabolite which was incubated with yeast phosphoriboisomerase. As

Figure 2 M.s. characterization of the ADPR metabolite

ADPR and its adenylic metabolite, purified by h.p.l.c. (phosphate and formate analysis) from erythrocyte cytosol incubated with ADPR, were analysed separately by h.p.l.c./m.s. Spectra obtained from the peaks at the retention times of ADPR (a) and its metabolite (b) are presented, followed by reconstructed chromatograms with the relevant m/z values in these spectra (c). Intensity is relative to the base peak (not shown in the Figure).

shown in Figure 4(a), h.p.l.c. analysis of the product demonstrated a peak with the same absorption spectrum and retention time as standard ADPR. In a parallel incubation, ADPR purified from the erythrocyte lysate was converted by phosphoriboisomerase to an adenylic peak co-eluted with the unknown metabolite (Figure 4b).

All these findings suggest that the adenylic metabolite produced upon incubation of ADPR with erythrocyte lysates is ADPRu. The recognition of ADPR by yeast phosphoriboisomerase suggests that the same enzyme activity is responsible for the isomerization of ADPR to ADPRu in the erythrocyte lysates as well. ADPRu had recently been identified as a metabolite in trichloroacetate extracts from human erythrocytes at a concentration of $0.11 \pm 0.01 \mu\text{M}$ [23].

Conversion of ADPR into the adenylic and inosinic nucleotide pools

Table 1 shows the metabolic conversion of ADPR in erythrocyte lysates at various times of incubation at 37 °C. In the absence of added ATP, ADPR was completely converted into the adenylic and inosinic pools within 3 h, whereas a minor amount of ADPR was rapidly transformed into ADPRu, which was eventually

Figure 3 N.m.r. spectra of ADPR and its metabolite

The 500 MHz proton spectra of ADPR recorded at 30 °C (a), the 500 MHz proton spectra of the ADPR metabolite recorded at 25 °C (b) and ^1H - ^{13}C proton-detected correlation spectroscopy of the ADPR metabolite recorded at 25 °C (c) are shown. The spectral widths were 4500 Hz and 22500 Hz for F2 and F1 respectively. For each of 256 experiments, 256 scans were recorded. Prior Fourier transformation data were zero-filled to give a final resolution of 2.2 Hz/pt in F2 and 44 Hz/pt in F1 (c). R = ribose ring: R1 (^1H 6.22 p.p.m., ^{13}C 90.4 p.p.m.); R2 (^1H 4.79 p.p.m., ^{13}C 76.8 p.p.m.); R3 (^1H 4.53 p.p.m., ^{13}C 72.7 p.p.m.); R4 (^1H 4.41 p.p.m., ^{13}C 86.8 p.p.m.); R5 (^1H 4.15 p.p.m., ^{13}C 66.5 p.p.m.). R' = ribulose fragment: R'1 (^1H 4.5/4.65 p.p.m., ^{13}C 69.5 p.p.m.); R'3 (^1H 4.40 p.p.m., ^{13}C 77.0 p.p.m.); R'4 (^1H 4.10 p.p.m., ^{13}C 73.0 p.p.m.); R'5 (^1H 3.96/4.15 p.p.m., ^{13}C 66.8 p.p.m.). The quaternary carbon shift (R'2) was not determined.

consumed. In the presence of 1 mM ATP, the rate of disappearance of ADPR was reduced considerably (20% still remained after 3 h), whereas the amount of ADPRu formed was higher and stable during incubation. However, even in the presence of ATP, the extent of isomerization of ADPR taking place in the haemolysate was much lower than its conversion into the adenylic and inosinic pools.

Figure 4 Incubation of yeast phosphoriboisomerase with ADPR and ADPRu purified from erythrocyte cytosol

Unidentified adenylic peak (5 nmol) (a) and 5 nmol of ADPR (b) purified from erythrocyte cytosol (see the Materials and methods section) were incubated at 37 °C with 10 µg of phosphoriboisomerase in PBS buffer. At 0 and 60 min, samples were extracted with trichloroacetic acid and subjected to phosphate h.p.l.c. —, Zero time; ····, 60 min.

Table 1 Conversion of ADPR into the adenylic and inosinic nucleotide pools

Haemolysate (1 ml) was incubated at 37 °C with 1 mM ADPR without or with 1 mM ATP. At the times indicated, 200 µl portions were extracted with 200 µl of 10% trichloroacetic acid and subjected to phosphate h.p.l.c. analysis. Results of a single representative experiment are shown for the sake of clarity. However, the range of variability of results in three different experiments never exceeded 15%. The adenylic pool is AMP + ADP + ATP. The inosinic pool is hypoxanthine + inosine + IMP.

Time (min)	Concentration (nmol/ml of cytosol)			
	ADPR	ADPRu	Adenylic pool	Inosinic pool
Incubation without ATP				
0	885	0	397	0
30	540	51	634	67
60	261	42	720	138
180	10	0	710	510
Incubation with 1 mM ATP				
0	967	0	1180	0
30	770	60	1313	33
60	710	88	1297	67
180	187	50	1248	603

In another experiment the haemolysate was incubated with [³²P]ADPR in order to identify the first catabolic product of ADPR, i.e. the nucleotide among the adenylic and inosinic pools with the highest rate of increase in specific radioactivity. As shown in Figure 5, the specific radioactivity of AMP was higher

Figure 5 Specific radioactivity of labelled nucleotides during the metabolic conversion of [³²P]ADPR in erythrocyte haemolysate

The haemolysate was incubated at 37 °C with 1 mM [³²P]ADPR. At the times indicated, samples were withdrawn and the trichloroacetate extracts were injected into the phosphate h.p.l.c. Fractions were collected every 30 s for determination of radioactivity. Results from a representative experiment are shown for the sake of clarity. Variability in four different experiments never exceeded 12%.

than those of ADP, ATP and IMP during the first 30 min of incubation. After 60 min incubation, when most of the ADPR had been degraded, the nucleotides in the adenylic pool had reached comparable specific radioactivities and this condition was maintained for the following 2 h. Thus, ADPR was degraded first to AMP, which was then converted into ADP, ATP and IMP. No radioactivity was detected as free phosphate throughout the incubation time, indicating that AMP was not dephosphorylated.

Another metabolite possibly arising from ADPR in erythrocyte lysates might be IDP-ribose (IDPR), as suggested by the deamination of ADPR to IDPR which is catalysed by a commercial adenylic acid deaminase [11]. However, we were unable to detect the presence of IDPR in haemolysates incubated with [³²P]ADPR under the conditions described in Figure 5 (the limit of detectability was 10 µM).

In contrast with erythrocyte lysates, erythrocyte membranes did not transform ADPR to AMP nor to ADPRu (not shown).

Encapsulation of [³²P]ADPR in human erythrocytes

In order to investigate the metabolism of ADPR in intact erythrocytes, this labelled nucleotide was encapsulated into erythrocytes by a procedure that produces no significant alterations in terms of biochemical and morphological properties of erythrocytes [24]. The data shown in Table 2 demonstrate that after 4 h incubation at 37 °C, 80 % of ADPR had disappeared. ADPRu present at zero time incubation (100 µM) was apparently produced during the 30 min time preceding the start of the experiment which was required for the encapsulation (haemolysis, resealing and washing of the resealed erythrocytes). During incubation of the resealed erythrocytes, the ADPRu concentration increased to 170 µM (after 2 h) and then decreased to undetectable values (after 7 h), indicating that both isomerization of ADPR and degradation of ADPRu took place in intact erythrocytes (Table 2).

The data concerning the intraerythrocytic concentrations of these adenine compounds indicate a metabolic pattern that is

Table 2 Encapsulation of [32 P]ADPR or [32 P]NAD $^{+}$ in human erythrocytes

[32 P]ADPR or [32 P]NAD $^{+}$ were encapsulated within erythrocytes by the procedure of hypotonic lysis and isotonic resealing. Erythrocytes were incubated at 37 °C at a 30% haematocrit in the presence of 1 mM adenosine, 1 mM nicotinamide and 5 mM glucose. At the times indicated, 200 μ l portions of washed erythrocytes were extracted with 150 μ l of 20% trichloroacetic acid. This was removed from the clear supernatant with diethyl ether, and concentration and specific radioactivity of the nucleotides were determined by phosphate h.p.l.c. on 25 μ l of the extract. Results from a representative experiment are shown for the sake of clarity. The range of variability in three different experiments was less than 12%. ND, not detectable.

Incubation (h)	Encapsulation of [³² P]ADPR						Encapsulation of [³² P]NAD ⁺	
	ADPR		ADPRu		AMP			
	(nmol/ml)	(c.p.m./nmol)	(nmol/ml)	(c.p.m./nmol)	(nmol/ml)	(c.p.m./nmol)	(nmol/l)	(c.p.m./nmol)
0	1344	9500	102	10400	114	2080	82.4	20700
2	449	10000	175	9800	213	4400	75.2	19600
4	278	10200	90	10000	175	4800	79.2	17600
7	ND	ND	ND	ND	162	4000	89.0	17100

closer to the transformations undergone by ADPR in haemolysates supplemented with ATP than in the same haemolysates without added ATP (see Table 1).

Throughout the incubation time (up to 4 h) the specific radioactivities of ADPR and ADPRu remained unchanged, whereas the specific radioactivity of AMP was doubled during the first 2 h incubation of the resealed erythrocytes (Table 2), thus confirming hydrolysis of the pyrophosphate bond of ADPR as the first reaction taking place inside the intact erythrocytes.

In a parallel experiment, [32 P]NAD $^{+}$ was encapsulated and its stability investigated, to provide a comparison with the metabolic conversions of ADPR. NAD $^{+}$ concentration was stable during the 7 h incubation and its specific radioactivity decreased slowly over time (20% in 7 h). Thus resynthesis of NAD $^{+}$ from unlabelled precursors prevented the decrease in the nucleotide concentration despite a 20% degradation during the 7 h incubation.

Partial purification and substrate specificity of the ADPR pyrophosphatase

The data obtained were clearly consistent with the presence, in the erythrocyte cytosol, of a dinucleotide pyrophosphatase activity responsible for the hydrolysis of ADPR to AMP (Figure

Figure 6 Labelling of erythrocyte membranes

Erythrocyte ghosts (300 μ g per lane) were incubated at 37 °C in 5 mM phosphate buffer, pH 8.0, with 2 mM MgCl $_2$ and the following additions: 10 7 c.p.m. [32 P]ADPR (6 $\times$ 10 7 c.p.m./nmol) (lane B); 10 7 c.p.m. [32 P]NAD $^{+}$ (same specific radioactivity) (lane C); 10 7 c.p.m. [32 P]NAD $^{+}$ with 1 mM ADPR (lane D); 10 7 c.p.m. [32 P]NAD $^{+}$ with 30 mM nicotinamide (lane E). After 60 min at 37 °C, samples were submitted to sideness experiments [18], showing that the ghosts were completely unsealed, and subjected to acid extraction (trichloroacetate followed by formic acid), SDS/PAGE and autoradiography as described in the Materials and methods section. Lane A, Coomassie staining. The protein bands are numbered as in Fairbanks et al [25]. Lanes B–E, autoradiographies.

Table 3 Substrate specificity of ADPR pyrophosphatase

Incubations were performed at 37 °C with 10 μ g of a 1080-fold purified enzyme preparation in 10 mM Tris/HCl, pH 8.3, with 1 mM substrate and 10 mM MgCl $_2$. At various times (0, 30, 60 min) of incubation, samples were deproteinized and injected into the h.p.l.c. system as described in the Materials and methods section. Results are expressed as percentage of activity on the various substrates compared with ADPR.

Substrate	Activity (%)
ADPR	100
ADPRu	64
NAD $^{+}$	42
NADH	21
NADPH	16
NADP $^{+}$	2
Cyclic ADPR	0
ATP	0
ADP-glucose	0
Inorganic pyrophosphate	0

5, Table 2). Indeed, a pyrophosphatase active on ADPR was partially purified from the erythrocyte cytosol by a two-step chromatography on DEAE-cellulose followed by NAD $^{+}$ -agarose (see the Materials and methods section). The final yield was 20% with a 1080–1200-fold purification, as calculated on the basis of the specific activity determined with 1 mM ADPR as substrate.

Table 3 shows that, among the nucleotides tested, ADPR and ADPRu were the preferred substrates of this enzyme. No activity was detectable in the absence of Mg $^{2+}$. This pyrophosphatase had no activity on inorganic pyrophosphate, cyclic ADPR and ADP-glucose. ATP inhibited enzyme activity by 50% when present at equimolar concentrations with ADPR (not shown).

Binding of ADPR to erythrocyte membranes and purified Ga3PDH

The lack of detectable transformations of ADPR incubated with erythrocyte membranes (see above) enabled us to investigate whether this metabolite could bind to membrane proteins.

Figure 7 Labelling of purified Ga3PDH

Ga3PDH, purified from human erythrocytes (250 μ g per lane), was incubated at 37 °C in 0.1 M Tris/HCl, pH 8.0, with the following additions: 3×10^7 c.p.m. of [*carbonyl*- 14 C]NAD⁺ (lane B); 3×10^7 c.p.m. of [*adenylate*- 32 P]NAD⁺ (lane C); 3×10^7 c.p.m. of [*adenylate*- 32 P]ADPR (lane D). After 15 min at 37 °C samples were subjected to acid extraction (trichloroacetic acid followed by formic acid), SDS/PAGE (10% acrylamide concentration) and autoradiography. Lane A, Coomassie staining. Lanes B–D, autoradiographies.

Indeed, on incubation of white unsealed erythrocyte ghosts with purified [32 P]ADPR (0.3 μ M, i.e. around its physiological concentration), several membrane proteins were labelled, although to markedly different extents (Figure 6, lane B). Specifically, label was detected on the spectrin region of the gel, on three proteins of 98, 79 and 72 kDa apparently co-migrating with bands 3, 4.1 and 4.2 respectively, on two other proteins of 58 and 41 kDa lacking any obvious correspondence with the Coomassie Blue-stained bands, and on a 36 kDa protein having the same mobility as Ga3PDH. The patterns of protein labelling by [32 P]ADPR were strictly comparable with those observed with [32 P]ADPRu [23]. In parallel experiments, purified spectrin was found to be labelled by [32 P]ADPR and the binding resisted treatment with trichloroacetate and formic acid (not shown). As shown in Figure 6 (lane C), the same proteins that bound [32 P]ADPR were also labelled when the erythrocyte ghosts were incubated with [32 P]NAD⁺, the two patterns being closely superimposable also in terms of amount of label on the various bands. Thus a low extent of labelling was observed for spectrin and the 58 and 41 kDa protein bands compared with that of the 98, 79, 72 and 36 kDa protein bands. On addition of excess unlabelled ADPR, or nicotinamide, to [32 P]NAD⁺ during the incubation, only the 98 kDa and the 36 kDa proteins were still labelled (Figure 6, lanes D and E respectively), although to a markedly lower extent than in the absence of both compounds.

Acid-resistant binding of free ADPR to Ga3PDH was confirmed by incubation of the enzyme purified from human erythrocytes with [32 P]ADPR (Figure 7, lane D). Under the same conditions of incubation and acid extraction (trichloroacetic acid followed by formic acid), purified Ga3PDH was also labelled by [*carbonyl*- 14 C]NAD⁺ and [*adenylate*- 32 P]NAD⁺ (Figure 7, lanes B and C respectively). Labelling of the enzyme with [*carbonyl*- 14 C]NAD⁺ was greatly enhanced when the incubation was performed in the presence of glyceraldehyde 3-phosphate (not shown).

The interaction of ADPR with Ga3PDH purified from human erythrocytes were also confirmed by a kinetic approach: the enzyme activity was inhibited by free ADPR in a competitive manner toward NAD⁺, the estimated K_i value being 0.25 mM.

Digestion of the labelled membrane proteins with snake venom pyrophosphatase

After incubation of erythrocyte membranes with [32 P]ADPR as in the experiment shown in Figure 6, the proteins were subjected

to SDS/PAGE and electroelution. The labelled proteins were incubated with or without snake venom pyrophosphatase as described in the Materials and methods section. The trichloroacetate precipitates were counted for radioactivity, and the supernatants were subjected to phosphate h.p.l.c. for identification of the released nucleotide. In the presence of the enzyme, no radioactivity was detectable in the trichloroacetate precipitate, and h.p.l.c. analysis of the supernatant identified labelled AMP as the only product of enzyme hydrolysis. In the absence of pyrophosphatase, all radioactivity remained in the trichloroacetate precipitate (not shown).

DISCUSSION

The present study concerned the patterns of intra-erythrocytic conversion of free ADPR, rather than addressing identification of its biosynthetic pathway(s). Previous experiments on incubation of intact erythrocytes with [32 P]orthophosphate have suggested a slow turnover of ADPR: incorporation of [32 P]P_i into ADPR was undetectable before 24 h incubation [11]. The experimental conditions described in Table 2 (7 h incubation of erythrocytes encapsulating [32 P]ADPR) and designed to investigate the intracellular degradation of ADPR were thus inadequate to evaluate a reduction in the specific radioactivity of ADPR due to synthesis. During the same time of incubation, however, a 20% decrease in the specific radioactivity of NAD⁺ occurred (Table 2). This demonstrates that the turnover of ADPR is at least slower than that of NAD⁺.

Possible sources of free ADPR might be hydrolysis of cyclic ADPR and of protein-linked ADPR. Cyclic ADPR, a potent Ca²⁺ mobilizer recently identified in several cell types [26–27], is hydrolysed to ADPR by a specific enzyme [28]. However, the presence in human erythrocytes of cyclic ADPR has not yet been described, although both enzymes involved in its formation and hydrolysis have been recently detected on the outer surface of erythrocytes [29]. On the other hand, the enzymes catalysing transfer of ADPR from NAD⁺ molecules and hydrolysis of ADPR from acceptor proteins have recently been purified from human erythrocyte cytosol [15–16]. In view of the demonstrated specificity of both enzymes, the α -subunit of G_i in the human erythrocyte membrane may be the physiological candidate for the generation of free ADPR. Nevertheless, the extent to which these reactions take place *in vivo* is open to investigation.

Figure 8 summarizes the metabolism of ADPR inside erythrocytes. As mentioned, indication of its sources is purely speculative: cyclic ADPR has not yet been detected in erythrocytes and specific mono-ADP-ribosylated proteins have not yet been identified. No substantial differences between erythrocyte cytosol and intact erythrocytes loaded with ADPR are apparent. In both cases, the major product of ADPR catabolism is AMP, which is rapidly phosphorylated to ADP and ATP by adenylate kinase and, to a lesser extent, deaminated to IMP by adenylate deaminase (Figure 5). The enzyme responsible for the cleavage of the phosphoanhydride bond of ADPR was partially purified from the erythrocyte cytosol; ADPR is the preferred substrate of this Mg²⁺-dependent enzyme (Table 3), which is inhibited by ATP. This inhibition explains the lower rate of ADPR consumption in haemolysates supplemented with ATP (Table 1). Two distinct ADPR pyrophosphatases have been identified and partially purified from rat liver [30], but to our knowledge this is the first demonstration of the existence of this enzyme in human erythrocytes.

The only other product of ADPR metabolism in erythrocyte lysates is ADPRu. This until now unknown metabolite of ADPR was identified by means of m.s. and n.m.r. spectroscopy (Figures

Figure 8 Metabolic pathways of ADPR in human erythrocytes

G_α-ADPR, ADP-ribosylated α-subunit of G_i.

2 and 3). ADPRu is also formed inside intact erythrocytes loaded with ADPR (Table 2). The observation that phosphoriboisomerase from yeast is capable of converting ADPR into ADPRu (Figure 4) suggests that this enzyme might also be responsible for the isomerization in human erythrocytes. As far as degradation is concerned, ADPRu is also a good substrate of the partially purified ADPR pyrophosphatase (Table 3), providing a likely mechanism for its time-dependent consumption inside erythrocytes (Table 2). The recent identification in our laboratory of ADPRu as normal metabolite in human erythrocytes [23] indicates that isomerization of ADPR is a physiological reaction in these cells.

Detection of free ADPR in human erythrocytes [11] prompted us to investigate its possible direct binding to erythrocyte membrane proteins by non-enzymic ADP-ribosylation. In preliminary experiments, membranes proved to be devoid of any ADPR-transforming enzyme activity. After incubation with [³²P]ADPR, the labelled membranes were precipitated with trichloroacetic acid, redissolved in formic acid and finally reprecipitated in trichloroacetic acid before SDS/PAGE analysis. This treatment is known to remove non-covalently bound nucleotides from acceptor proteins [19]. Figure 6 (lane B) shows that several membrane proteins are labelled on incubation of erythrocyte ghosts with [³²P]ADPR.

Identification of these proteins with specific integral and cytoskeletal membrane proteins will be the subject of future work. In fact, such identification requires immunological or sequence-analysis criteria, or the use of selective procedures by which different sets of proteins can be extracted from erythrocyte membranes [31–33]. Nonetheless, alignment of the autoradiograms with the Coomassie Blue-stained gels indicates correspondence of the ADP-ribosylated bands with spectrin, bands 3, 4.1 and 4.2 and Ga3PDH. In contrast, the two labelled bands of 58 and 41 kDa do not correspond to any Coomassie Blue-stained bands.

Of these proteins, purified spectrin (not shown) and Ga3PDH (Figure 7) were labelled on incubation with [³²P]ADPR and can therefore reasonably be identified as acceptors of ADPR within the erythrocyte membrane. It should be noticed that ADPR fails to bind to other membrane proteins (e.g. ankyrin, actin, band 7), indicating some selectivity in its interaction with erythrocyte membrane proteins. Closely comparable patterns of [³²P]ADPR binding were still observed on lowering the pH from 8.0 to 7.0

(not shown). The extent of Ga3PDH ADP-ribosylation was quite low. Determination of the radioactivity of the sample subjected to SDS/PAGE (Figure 7, lane D) enabled us to calculate the stoichiometry of ADPR binding to be 2–4 mmol of ADPR/mol of Ga3PDH. However, the low stoichiometry of modification by labelled ADPR *in vitro* might reflect a large extent of pre-existing ADP-ribosylation (and of tightly bound NAD⁺ as well) in the intact erythrocytes or after preparation of white ghosts.

The same proteins that were labelled after exposure of the membranes to [³²P]ADPR were also labelled on incubation of erythrocyte ghosts with radioactive NAD⁺ (Figure 6, lane C). However, addition of ADPR (Figure 6, lane D) or nicotinamide (Figure 6, lane E) to the incubation mixture led to a decrease in the radioactivity of the 98 kDa protein and Ga3PDH and to the almost complete disappearance of all other bands. These results strongly suggest that, during incubation with unsealed ghosts, [³²P]NAD⁺ was first converted into [³²P]ADPR by membrane-bound NAD⁺ glycohydrolase and [³²P]ADPR then bound to the proteins.

Particular attention was devoted to the interaction of Ga3PDH and ADPR. The enzyme purified from human erythrocyte membranes were labelled on incubation with [³²P]ADPR (Figure 7, lane D), which extends an earlier observation on the enzyme from rat muscle [8]. The purified enzyme was also labelled on incubation with [¹⁴C]NAD⁺ as well as with [adenylate-³²P]NAD⁺ (Figure 7, lanes B and C). Thus binding between NAD⁺ and Ga3PDH, as well as that between ADPR and Ga3PDH, is so tight as to resist treatment with trichloroacetic acid and formic acid.

These results suggest caution in the interpretation of the several experiments designed to evaluate enzyme-catalysed ADP-ribosylation of Ga3PDH in erythrocytes. Recently, a nitric oxide-stimulated NAD⁺-dependent auto-ADP-ribosylation of erythrocyte Ga3PDH was described [34–36]. Kots *et al.* [34] were unable to detect labelling of Ga3PDH using [carbonyl-¹⁴C]NAD⁺, instead of [adenylate-³²P]NAD⁺, but their data do not allow us to conclude whether the failure to observe this modification applied to the purified enzyme or to the same protein in the erythrocyte membrane. In the latter case, the NAD⁺ glycohydrolase activity present on the membrane could easily have converted all available NAD⁺ to unlabelled ADPR. On the other hand, the only reagent used by Dimmeler *et al.* [35] and by Zhang and Snyder [36] to label purified Ga3PDH in the presence of nitric oxide was [adenylate-³²P]NAD⁺; these authors could therefore hardly distinguish between ADP-ribosylation and binding of NAD⁺ to the enzyme.

Clearly, the erythrocyte seems to represent at best a difficult cell type for studies on auto-ADP-ribosylation of Ga3PDH. In fact NAD⁺ glycohydrolase activity on the erythrocyte membrane can generate free ADPR, which in turn is able to bind to Ga3PDH in unsealed membranes (see above). In addition, the acid-resistant binding of NAD⁺ itself to the erythrocyte Ga3PDH (Figure 7, lanes B and C), particularly in the presence of its substrate, makes it impossible to discriminate between binding of NAD⁺ to the protein and NAD⁺-dependent auto-ADP-ribosylation by using [³²P]NAD⁺ only.

The strong binding of free ADPR to Ga3PDH prompted us to investigate the effect of this apparently covalent modification on enzyme activity. Indeed, ADPR exerted a competitive inhibition toward NAD⁺, with a K_i of 250 μM (not shown). This high K_i value was estimated for the enzyme extracted and purified from solubilized erythrocyte ghosts [17]. In fact, a significant fraction of this enzyme protein is closely bound to the erythrocyte membrane [17] where, or in proximity of which, ADPR might be

formed. This close interaction in the erythrocyte could affect the kinetic data significantly, making the inhibition of Ga3PDH activity by free ADPR physiologically relevant.

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