Chemical and Metabolic Properties of Adenosine Diphosphate Ribose Derivatives of Nuclear Proteins

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1. ADP-ribose is found in rat liver nuclei covalently bound to histone F1, to a non-histone protein, and to a small peptide. 2. A single unit of ADP-ribose, covalently bound to phosphoserine, was isolated from an enzymic hydrolysate of histone F1. ADP-ribose-bearing peptides were isolated from a tryptic digest of the histone. 3. It is proposed that the 1'-hydroxyl group of ADP-ribose is linked to the phosphate group of phosphoserine in histone F1. 4. The incorporation of ^{32}P into ADP-ribose on histone F1 parallels the DNA content through the cell cycle. An increased incorporation of the nucleotide into the other derivatives is observed during S phase. 5. It is suggested that the ADP-ribose derivative of histone F1 has a role in maintaining the G_0 state and that one or both of the other derivatives is concerned with control of DNA synthesis.

Histone F1 carries phosphate as phosphoserine (Ord & Stocken, 1966), as phospholysine (Chen *et al.*, 1974) and as covalently bound ADP-ribose *in vivo* (Smith & Stocken, 1973a). ADP-ribose has also been noted, as ^3H radioactivity, in various protein extracts from nuclei which have been incubated *in vitro* with [^3H]adenosine-labelled NAD^+ (Nishizuka *et al.*, 1968; Otake *et al.*, 1969; Burzio & Koide, 1971; Ueda *et al.*, 1972; Koide & Burzio, 1972; Dietrich & Siebert, 1974). Some indication that the ADP-ribose might be covalently bound is provided by Nishizuka *et al.* (1968), who remarked on the tenacity of ADP-ribose binding, by Ueda *et al.* (1972), who found that trypsin treatment of nuclear extracts after the incubation of nuclei with labelled NAD^+ changed the elution pattern from Sephadex, and by Nishizuka *et al.* (1969) and Dietrich & Siebert (1974), who found that neutral hydroxylamine released labelled ADP-ribose from nuclei incubated with NAD^+ .

In the present paper, the bond between ADP-ribose and histone F1 is characterized, and the identification of other protein acceptors of ADP-ribose is reported. Evidence for the function of ADP-ribose derivatives of nuclear proteins is discussed.

Materials and Methods

Animals

Male Wistar rats (body wt. 190 g; this laboratory's strain) were partially hepatectomized (Higgins & Anderson, 1931) under ether anaesthesia between 11.00 and 13.00 h.

Labelling with ^{32}P *in vivo*

The animals were injected intraperitoneally with

125 μCi of carrier-free [^{32}P]orthophosphate/100 g body wt. in 0.3 ml of 0.9% NaCl, 90 min before being killed.

Labelling with [^3H]adenosine-labelled NAD^+ *in vitro*

Nuclei derived from the livers of six rats were suspended in 5 vol. of 10 mM-Tris-HCl buffer, pH 7.4, containing 28 mM-MgCl₂, 1 mg of glucose/ml and 25 μCi of [^3H]adenosine-labelled NAD^+ for 10 min at 37°C.

Preparation of nuclei

Nuclei were prepared by the method of Chauveau *et al.* (1956).

Preparation and purification of histone F1

Nuclei were washed with 10 mM-Tris-HCl buffer, pH 7.4, containing 5 mM-MgCl₂, and histone F1 was extracted with 5% (w/v) HClO₄. The extract was made 20% (w/v) with respect to trichloroacetic acid, and centrifuged at 3000g for 10 min (Johns, 1964). The precipitated histone was dissolved in water and separated from contaminating non-histone proteins by chromatography on a DEAE-cellulose column (Buckingham & Stocken, 1970). The product gave a single band when electrophoresed on polyacrylamide gel as described by Panyim & Chalkley (1969) or MacGillivray *et al.* (1972).

Extraction of nuclear proteins

After extraction of histone F1, the nuclear residue was extracted with 0.25 M-HCl to remove the remaining histones. Non-histone proteins were extracted by the method of Gronow & Griffiths (1971).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out at pH2.9 by the method of Panyim & Chalkley (1969) and at pH8.9 by the method of Davis (1964).

Amino acid analysis

The method of Spackman *et al.* (1958) was used. Analyses were performed on a Locarte amino acid analyser, a single column being used for acidic, neutral and basic amino acids.

Ribose determination

Ribose was determined by the method of Hurlbert *et al.* (1954).

Phosphate determination

Phosphate was determined by the method of Bartlett (1959) with 60% (w/v) HClO_4 as the ashing agent.

Adenine determination

Adenine was determined by t.l.c. in the solvent of Lane (1963) and determined by its absorbance at 260 nm.

ADP-ribose

ADP-ribose was identified by chromatography on a column of Dowex 1 (formate form) (Nishizuka *et al.*, 1967), and by paper chromatography in isobutyric acid–conc. NH_3 (sp. gr. 0.880)–water (66:1:33, by vol.) containing EDTA (0.1 mM) (Reeder *et al.*, 1967).

Separation of nucleotides on the basis of chain length

ADP-ribose oligomers bound to histone F1 were separated according to their size by chromatography of an enzymic hydrolysate of the histone on a column of DEAE-Sephadex, by the method of Seno *et al.* (1968).

Enzymic digestion of histone F1

Complete enzymic hydrolysis of histone F1 was carried out with Pronase and leucine aminopeptidase by the method of Balhorn *et al.* (1971). Digestion with trypsin was carried out by the method of Sung *et al.* (1971).

Electrophoresis of peptides

A tryptic digest of histone F1 was applied to Whatman 3MM paper and electrophoresed for 1 h at 3.5 kV at pH6.5 in pyridine–water–acetic acid (10:89:0.4, by vol.). Orange G was used as marker.

Enzymes

Trypsin, treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one to inhibit chymotryptic activity, was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pronase, derived from *Streptomyces griseus*, was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Leucine

aminopeptidase, type III CP, derived from pig kidney, was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Radioactive isotopes

^{32}P Orthophosphate with specific radioactivity 67 Ci/mg of P was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ^3H Adenosine-labelled NAD^+ was obtained from NEN Chemicals G.m.b.H., Dreieichenheim, W. Germany. Its specific radioactivity was 5 Ci/mmol.

Results

ADP-ribose associated with histone F1

Our previous results (Smith & Stocken, 1973a) showed that ADP-ribose is present covalently bound to histone F1 *in vivo* and that the average chain length of the oligonucleotide was about three ADP-ribose units. Nothing was said, however, about the size distribution of the nucleotide. To answer this question, histone F1 was prepared from rat liver which had been labelled with ^{32}P *in vivo*. It was hydrolysed completely with Pronase and leucine aminopeptidase and examined by chromatography on DEAE-Sephadex (Fig. 1). After elution of the run-off peak, which contained the bulk of the amino acids, including phosphoserine, most of the radioactivity was

Fig. 1. Separation of the products of an enzymic hydrolysate of histone F1 on DEAE-Sephadex

Rat liver histone F1, labelled with ^{32}P *in vivo*, was digested with Pronase and leucine aminopeptidase. It was applied to a column of DEAE-Sephadex (A-25) (20 cm $\times$ 3 cm²) and eluted by a salt gradient in the presence of 7 M-urea. The arrow indicates the position at which the column was washed with 7 M-urea–0.7 M-NaCl. All solutions were buffered at pH7.4 with 20 mM-Tris–HCl.

found in the fractions expected to contain the serine-bound ADP-ribose monomer. Much of the remaining radioactivity was not recovered until the column was washed with buffer containing 0.7 M-NaCl, and represents a polymer consisting of more than six ADP-ribose units.

The fractions containing the presumed monomer were diluted fivefold and applied to a small column of DEAE-Sephadex (A-25, 2 cm × 1 cm²). The column was washed with 10 vol. of water, and the nucleotide eluted with 1 M-NH₄HCO₃ and freeze-dried. Analysis of the material so obtained (Table 1) gave the proportions serine/adenine/ribose/phosphate = 1:1:2:3. Mild acid hydrolysis (0.3 M-HCl for 5 min at 30°C) gave phosphoserine and ADP-ribose. The structure shown in Fig. 2 is proposed for the seryl-phosphoryl-ADP-ribose derived from histone F1.

Location of the nucleotides

To locate the serine moiety carrying the ADP-ribose on the histone F1 molecule, a tryptic digest of histone

Table 1. Analysis of material from an enzymic hydrolysate of histone F1 eluted from DEAE-Sephadex in the monomer position

Each value is the mean of duplicate determinations and is expressed as nmol/mg of histone.

	Expt. I	Expt. II
Serine	1.89	1.77
Ribose	4.40	3.33
Phosphate	6.01	5.37
Adenine	1.86	1.74
Serine phosphate	Not determined	1.80

F1, labelled *in vivo* with ³²P, was subjected to high-voltage electrophoresis on paper at pH 6.5. The phosphate-containing tryptic peptides (A and B, Fig. 3) were well separated from the bulk of the peptides and had a u.v.-absorption maximum at 260 nm. Their amino acid analyses are shown in Table 2. They appear to be variations of a single sequence. The amino acid contents of these peptides do not correspond to any part of the published sequence of a sub-fraction of rabbit thymus histone F1 (Jones *et al.*,

Fig. 3. Paper electrophoretogram of tryptic digest of histone F1

Rat liver histone F1, labelled with ³²P *in vivo*, was digested with trypsin. It was subjected to paper electrophoresis at pH 6.5. The peptides taken for amino acid analysis are indicated with arrows. The Orange G marker ran 21 cm towards the anode.

Fig. 2. Proposed structure for the phosphoseryl poly(ADP-ribose)

Table 2. Amino acid analysis of tryptic peptides of histone F1 which contain ADP-ribose (A and B of Fig. 3)

Values are molar proportions, calculated relative to serine (=2). Serine and threonine values are corrected for degradation during hydrolysis. During hydrolysis adenine is degraded to give glycine equivalent to 0.5mol/mol of adenine. Values in parentheses indicate probable numbers of residues per peptide.

	A	B
Asp	2.0 (2)	3.1 (3)
Thr	1.2 (1)	1.3 (1)
Ser	2.0 (2)	2.0 (2)
Glu	2.9 (3)	4.1 (4)
Gly	2.3 (2)	2.3 (2)
Ala	3.0 (3)	2.1 (2)
Val	0.9 (1)	0.8 (1)
Leu	0.9 (1)	0.8 (1)
Lys	1.0 (1)	0.8 (1)

1974). The possibility that one of the known phosphopeptides (Langan, 1969; Dixon *et al.*, 1973) also carries ADP-ribose is not ruled out, as much of the nucleotide remained at the origin of the electrophoretogram, and could not therefore be analysed, owing to the presence of contaminating peptides.

Content of ADP-ribose on histone F1 *in vivo*

Since the proposed ribose 1-phosphate bond is acid-labile (Kalckar, 1945) it seemed likely that some of the ADP-ribose was split off during extraction with HClO_4 . To minimize the loss, histone F1 was extracted at 4°C from rat liver nuclei with 0.6M-NaCl-10mM-Tris-HCl at pH7.4, and the precipitation with 20% trichloroacetic acid carried out in the shortest possible time (not more than 20min at 4°C). The non-histone proteins present in the extract were separated by means of DEAE-cellulose chromatography under the same conditions as used for acid-extracted histone F1. The total phosphate content of the histone was 0.24mol/mol of protein, of which only 0.02mol/mol of protein was unsubstituted phosphoserine.

Other ADP-ribose acceptors

The 5% HClO_4 extract contains, in addition to histone F1, a non-histone protein P1 (Smith & Stocken, 1973b) and a small peptide of molecular weight about 3000. This small peptide is not precipitated in 20% trichloroacetic acid, as are histone F1 and protein P1, but can be obtained by the removal of trichloroacetic acid with ether, and precipitation with 2 vol. of ethanol. It was purified as described by Islam & Kay (1972) for their 'nucleotide-peptide'. The product ran as a single band close to the Bromophenol Blue marker dye on polyacrylamide-gel electrophoresis at pH8.9. The band stained faintly

with Naphthalene Black and contained 80% of the ^{32}P radioactivity put on the gel. The stained part of the gel was hydrolysed with 60% (w/v) HClO_4 for 30min at 100°C, and adenine was found in the hydrolysate. The amino acid analysis of the peptide is shown in Table 3, and on average 1 molecule of adenine is associated with 25 amino acid residues. ADP-ribose was identified in the alkaline digest of the peptide by chromatography on a Dowex-1 (formate form) column. When the peptide was prepared from nuclei which had been incubated *in vitro* with [^3H]adenine-labelled NAD^+ , ^3H radioactivity migrated with the peptide on polyacrylamide-gel electrophoresis.

ADP-ribose was also found in the 0.25M-HCl extract after removal of the histone F1 fraction with HClO_4 . Nuclei were incubated with [^3H]NAD $^+$ and the total histone minus the F1 fraction was chromatographed on Sephadex G-200 with 20mM-Tris-HCl buffer, pH7.4, containing 8M-urea. Under these conditions the protein carrying ADP-ribose was totally excluded from the Sephadex. The amino acid analysis is shown in Table 3. When this ADP-ribose-associated protein was rechromatographed on Sephadex G-200, in the same solvent made 0.2% with respect to sodium dodecyl sulphate, a small amount was eluted in the void volume, but the major portion was in a peak corresponding to a molecular weight of about 40000, with the amino acid analysis shown in column 3, Table 3. Non-histone proteins prepared from nuclei incubated with [^3H]NAD $^+$ were also chromatographed on Sephadex G-200 with 20mM-Tris-HCl buffer, pH7.4, containing 8M-urea. The protein carrying ADP-ribose was totally excluded from the Sephadex. When this ADP-ribose-associated protein was rechromatographed on Sephadex G-200 in the presence of 0.2% sodium dodecyl sulphate, the major portion was in a peak corresponding to a molecular weight of about 40000 and having the amino acid analysis given in column 4, Table 3.

ADP-ribosylation through the cell cycle

The changes in incorporation of ^{32}P radioactivity into ADP-ribose derivatives after partial hepatectomy were studied. For convenience, a simple method for separating the ADP-ribose derivatives was required. For histone F1, the method used was the one by which Balhorn *et al.* (1971) separated contaminating nucleotide from the histone on an Amberlite IRC 50 column. If the extensive dialysis against acid is omitted, that part of the histone F1 that carries the nucleotide is eluted first, and the histone that is retarded by the column consists of unsubstituted or simply phosphorylated histone. The ADP-ribosylated histone F1 is designated histone F1a and the remainder histone F1b. After partial hepatectomy, the incorporation of [^{32}P]phosphate into histone F1a

Table 3. *Amino acid analysis of proteins associated with ADP-ribose*

Proteins were prepared as described in the text. Values are expressed as mol %.

	'Nucleotide peptide'	'Total histone' excluded from Sephadex G-200	Aggregated material re-run on Sephadex G-200 in the presence of sodium dodecyl sulphate	
			From 'total histone minus F1'	From 'non-histone'
Asx	13.05	6.21	7.97	8.12
Thr	4.37	4.52	3.89	3.06
Ser	16.30	18.40	22.80	24.01
Glx	14.74	14.29	13.84	14.31
Pro	1.58	3.23	2.36	2.30
Gly	24.17	18.12	19.71	19.52
Ala	8.45	9.30	10.41	11.32
Cys	0	0	0	0
Val	3.87	3.78	0.52	0.77
Met	0	0.61	0.78	1.52
Ile	2.38	2.46	1.52	1.54
Leu	4.44	3.93	2.35	2.36
Tyr	0	1.59	0.52	0.76
Phe	0	1.72	1.10	0.78
His	2.24	3.62	4.04	3.10
Lys	3.97	4.60	1.80	4.13
Arg	0.67	3.62	1.86	2.39

remained constant, whereas incorporation into histone F1b, the amount of which was very low in resting liver, showed a small early rise followed by a second larger peak during S phase (Fig. 4). The early increase of ^{32}P incorporation into histone F1b was accompanied by a decrease in the relative amount of protein F1a to F1b.

The other ADP-ribose derivative studied in this way was the small peptide, precipitated by ethanol. In this case simple phosphorylation could not be detected. In contrast with the pattern with histone F1a, the incorporation into ADP-ribose on this peptide (see above) showed an early increase which continued until the S phase, when a maximum was reached (Fig. 5a).

The incorporation of ^{32}P during the cell cycle into the ADP-ribose associated with the other non-histone proteins has not been studied in detail, but we noted that the amount of ribose associated with the 'total histone minus histone F1' increased from 2.7 nmol of ribose/mg of protein in resting liver to 4.8 nmol of ribose/mg of protein 21 h after partial hepatectomy.

Discussion

The available information about chemically synthesized bonds between nucleotide and peptide material has been reviewed by Shaborova (1970). The compounds most similar to the proposed seryl-phosphoryl-ADP-ribose are those in which the hydroxyl group of serine is esterified with the phosphate

of 5'-UMP. This phosphodiester linkage is found to be alkali-labile, although if the amino group of the serine is free, the phosphate and its attached nucleoside can migrate to form the more alkali-stable phospho-amide derivative (Yuodka *et al.*, 1968). The phosphodiester bond is stable to neutral hydroxylamine (Berkowitz & Bendich, 1965; Yuodka *et al.*, 1969). It is fairly acid-stable, particularly in the absence of neighbouring hydroxyl groups, but breaks down slowly in 1 M-HCl at 37°C to give the nucleotide and O-phosphoserine. The structure proposed in Fig. 2, however, contains a ribose 1-phosphate bond which is extremely acid-labile. Ribose 1-phosphate itself is hydrolysed by 0.3 M-HCl at 30°C with a half-life of 1 min (Kalckar, 1945). The 1-phosphate is slightly stabilized when a 5-phosphate is also present (Klenow, 1953). The proposed linkage should be sufficiently stable to survive rapid extraction with HClO_4 . It would be expected to have alkali-lability and be hydroxylamine-stable.

The lability to hydroxylamine of some of the ADP-ribose associated with the non-histone proteins, which is found by Nishizuka *et al.* (1969), Ueda *et al.* (1972), Dietrich *et al.* (1973) and Dietrich & Siebert (1973), is of the same order as that found for the aminoacyl-tRNA bond, and has led to the suggestion that a similar bond exists between a carboxyl group of the protein and a hydroxyl group of ADP-ribose. Where all the ADP-ribose is found to be hydroxylamine-labile, the proteins have first been dialysed against acid (Nishizuka *et al.*, 1969), so both types of bond could be present in the non-histone derivatives.

Bruegger *et al.* (1974) have isolated 3-phosphohistidine and ϵ -phospholysine from histone digests. These compounds are extremely acid-labile and, if the phosphate group acts as a point of attachment for ADP-ribose, could provide another explanation of hydroxylamine lability.

The incorporation of ^{32}P into the ADP-ribose on histone F1a during a 90 min pulse at different times after partial hepatectomy roughly parallels the DNA content, as was found by Hilz & Kittler (1971) for the activity of their ADP-ribose polymerase. However, it must be noted (Ord & Stocken, 1975) that the total amount of ADP-ribose on histone F1 declines as the cell moves into S phase and rises again in G2 phase. Of the other acceptors, the ^{32}P radioactivity of the small ADP-ribose peptide is high during the S phase, whereas the ADP-ribosylation of protein P1 (Ord & Stocken, 1975) occurs at the G1/S phase transition and declines to base values at the end of the S phase.

Fig. 4. Change in incorporation of ^{32}P radioactivity after partial hepatectomy

Incorporation of ^{32}P radioactivity was measured (a) into histone F1b, (b) into histone F1a and (c) as a percentage of ^{32}P radioactivity in histone F1 separating into histone F1b. Each point is the average $\pm$ S.E.M. for four animals.

Fig. 5. Change in incorporation of ^{32}P radioactivity after partial hepatectomy

Incorporation of ^{32}P radioactivity was measured (a) into ADP-ribose-carrying peptide and (b) into DNA. Each point is the average $\pm$ S.E.M. for four animals.

What particular function is to be ascribed to ADP-ribosylation is uncertain. Just as phosphorylation has been postulated as a mechanism for extending (Louie *et al.*, 1973) or condensing (Bradbury *et al.*, 1974) chromatin, so too can ADP-ribosylation. The ADP-ribose moiety, by introducing a cluster of negative charges into the highly basic C-terminal of the molecule, might be a means of weakening the histone F1-DNA interactions in transcriptionally active regions. Yoshihara & Koide (1973) have suggested that poly-(ADP-ribose) releases DNA polymerase from chromatin, and it may be that the ADP-ribosylation of histone F1 and/or the ethanol-insoluble peptide is responsible for the termination of DNA synthesis (Ord & Stocken, 1975).

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