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ORIGINALARBEITEN

A note on the ADP-ribose-protein linkages in rat-liver nuclei: a possible approach to assessing megavitamin therapy with niacin

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Summary

Nuclei isolated from rat liver were incubated with $NAD^{\oplus}$ whose two ribose moieties were respectively labeled with ^{3}H or ^{14}C. By enzymatic (phosphodiesterase) and/or chemical (hydroxylamine) attack on doubly labeled ADP-ribosylated nuclear proteins and identification of fragments released from nuclear residues, AMP was found after hydroxylaminolysis as well as iso-ADP-ribose after phosphodiesterase plus hydroxylamine, in the absence of detectable amounts of ribose-5-phosphate. This is taken to indicate the existence of additional ribose-protein binding sites in in vitro ADP-ribosylated nuclear proteins: Besides C-1″ (Hayaishi et al., Stocken et al.) C-2′ and/or C-3′ (purine-near) as well as C-2″ and/or C-3″ (pyrimidine-near), not only at the end but also within the chain of oligo-ADPR.

Zusammenfassung

Aus der Rattenleber isolierte Zellkerne werden mit $NAD^{\oplus}$ inkubiert, dessen beide Ribosen mit jeweils ^{3}H oder ^{14}C markiert sind. Enzymatischer (Phosphodiesterase) und/oder chemischer (Hydroxylamin) Abbau markierter ADP-ribosylierter Proteine und Identifizierung der aus Zellkernrückständen freigesetzten Bruchstücke erweist, daß AMP nach Hydroxylaminolyse sowie iso-ADP-Ribose nach Phosphodiesterase und Hydroxylamin – bei Fehlen nachweisbarer Mengen an Ribose-5-phosphat – das Vorkommen zusätzlicher Bindungsformen zwischen Protein und Ribose in in vitro ADP-ribosylierten Zellkern-Proteinen verlangen: Neben C-1″ (Hayaishi u. a., Stocken u. a.) noch C-2′ und/oder C-3′ (Purin-nahe) und weiterhin C-2″ und/oder C-3″ (Pyrimidin-nahe), und dies nicht nur am Kettenende, sondern auch in der Mitte der Kette von Oligo-ADPR.

Key words: ADP-ribose, liver, nuclei, NAD, protein-ADP ribosylation, ribose-protein bonds

Introduction

In previous papers, consequences of excessive administration of nicotinamide have been described at the $NAD^{\oplus}$ level (1) and in regard to ADP-ribose protein conjugates (2). Since the discovery of ADP-ribose in covalent linkage to nuclear proteins by Chambon et al. (3), several efforts have been reported to identify the chemical nature of the covalent bond between nucleotide and protein (for review, see Purnell et al. (4)). Whereas at the protein site a considerable number of amino acid residues has been invoked, it is essentially the work of Hayaishi et al. (5) in which C-1″ of the

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pyridine-near ribose (in chain-end position) has been described as giving rise to ADP-ribosylation. More recently, information is increasing on the multiplicity of the type of protein-ADPR conjugates (6).

The differential stability of bound ADP-ribose towards alkali and/or neutral hydroxylamine (7–9) and supposed existence of a third alkali-resistant bond in vivo (10), as well as some preliminary observations (1) led to a reassessment, with the aid of doubly labeled $NAD^{\oplus}$ as precursor, of the chemical identity of the ADPR component taking part in the covalent bond to protein(s). It is shown in this communication that more than one type of covalent bond is formed in ADP-ribosylated nuclear proteins of rat liver in vitro.

Materials and methods

Enzymes (alkaline phosphatase, phosphodiesterase from snake venom, NAD pyrophosphorylase) were from Boehringer, Mannheim or Merck, Darmstadt (proteinase K, phosphodiesterase from snake venom). Calbiochem, San Diego, supplied ADP-ribose, Amersham-Buchler, Braunschweig, [U-^{14}C]-adenine-NAD with 100–200 mCi/mmol, [U-^{14}C]-adenosine-5′-triphosphate with 529 mCi/mmol, and [5-^{3}H]-D-ribose with 9.6 Ci/mmol, NEN Chemicals, Dreieichenhain bei Frankfurt/M., radioactive standards (water-^{3}H and toluene-^{14}C) and Biofluor as cocktail for LSC. Nucleosides and nucleotides were from Boehringer, Mannheim, also reagents for enzymatic analyses; reagent grade chemicals from Merck, Darmstadt, Dowex 1X8, 200 mesh, pract. from Serva, Heidelberg.

Radioactivity was measured in a liquid scintillation counter (Beckman Instruments, München); the yield was obtained from internal standards. Thin layer chromatograms and paper electrophoretic runs were counted with a thin-layer counter (Berthold, Wildbad).

Labelled NAD's were prepared as follows: Rats received [5-^{3}H]-D-ribose (11), and tritium-labeled NAD was isolated according to Brumm and Potter (12). [^{3}H-ribose]-NMN was obtained by the action of phosphodiesterase or NAD pyrophosphorylase. Resynthesis of [^{3}H-NMN]-NAD with exclusive label in the pyridine-near ribose was performed according to (13). [^{14}C-Ado]-NAD with exclusive label in the purine-near ribose was synthetized from [U-^{14}C]-adenosine-5′-triphosphate and NMN. Specific radioactivities are based on enzymatic analysis (14) and gave 12.51 μCi/mol for [^{14}C-adenosine]-NAD, respectively 0.75 μCi/μmol, for [^{3}H-ribose in NMN]-NAD. The purity of the NAD's was checked by i) rechromatography on Dowex 1X8 with a discontinuous gradient (15), ii) uv spectra in 1 M KCN (16), iii) enzymatic analysis (14) and comparison with uv absorption, and iv) finally thin-layer chromatography (see below) in solvent # 1 and radioactivity measurement as described. $NAD^{\oplus}$ (β-nicotinamide-adenine-dinucleotide; free acid, grade I, 100 %, from Boehringer) served as reference.

Nuclei were isolated from the liver of female SIV-50 albino rats (Ivanovas animal farm, Kißlegg), of 150–200 g body weight, according to Blobel and Potter (17). Incubation of nuclei corresponding to 20 g fresh rat liver was in 0.05 M Tris/Cl^{-} of pH 8.2, 0.014 M $MgCl_2$ and 0.037 M KCl with the following additions: 5×10^{-4}M NAD with 1.45×10^{6} dpm/μmol for [^{14}C-Ado]-NAD, 1.32×10^{6} dpm/μmol for [^{14}C-Ad]-NAD,

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Abbreviations

ADPR = adenosinediphosphoribose; *AMP* = adenosine-5′-monophosphate; *(^{14}C-Ad)-NAD* = (U-^{14}C)-adenine-NAD; *(^{14}C-Ado)-NAD* = (U-^{14}C)-adenosine-NAD; *(^{3}H-NMN)-NAD* = NAD-(ribose in NMN)-^{3}H; *iso-ADPR* = 2′-(5″-phosphoribosyl)-adenosinemonophosphate; *NAD* = β-nicotinamide adenine dinucleotide; *oligo-ADPR* = oligo-(adenosinediphosphoribose)$_n$; $n \leqq 10$.

and 1.45×10^6 dpm/μmol for the mixture of [^{14}C-Ado] and [^{3}H-NMN] NAD. After 10 min at 25 °C the incorporation was stopped with ice-cold perchloric acid at 3 % final concentration.

Phosphodiesterase digestion of labeled nuclear precipitate was performed after washing of the nuclear precipitate with 3 % perchloric, acid, until no more radioactivity could be removed, followed by 3 washings with 0.05 M Tris/Cl^- of pH 7.0. The near-neutral washed residue was suspended in 2 ml 0.2 M Tris/Cl^- of pH 7.4 and incubated for 3 hrs at 25 °C with 50 μg enzyme/ml. The incubation was stopped as above. The extent of phosphodiesterase digestion was frequently checked by a second enzymatic attack on the residue; in the average, more than 90 % of total solubilized radioactivity was obtained after the first incubation. The neutralized supernatant was chromatographed on Dowex 1X8.

Treatment with hydroxylamine or alkali. The washed nuclear residue after phosphodiesterase incubation was kept for 1 hr at 0 °C in 0.4 M NH_2OH, pH 7.0, or in 0.2 N NaOH, respectively. The reactions were stopped with $HClO_4$ and chromatographed as above.

Thin-layer chromatography was on cellulose plates (Merck, Darmstadt) with fluorescent indicator in the following solvents:

1 isobutyric acid:conc. NH_3:H_2O = 66:1.7:33 (v/v/v) (16)

2 0.1 M sodium phosphate, pH 6.8:ammonium sulfate:1-propanol = 100:60:2 (v/w/v) (16)

3 H_2O:1-propanol:conc. NH_3 = 25:55:30 (v/v/v) (18).

For localization, uv light, aniline phthalate (19), and Hanes reagent (20) were used.

Paper electrophoresis was done on Schleicher & Schüll No. 2043 ag., washed, in 0.1 M borate buffer, pH 9.2 (21) or in 0.05 carbonate buffer, pH 9.2, for 12–13 hrs at 100 Volt.

Results and discussion

Besides [^{14}C-adenine]-NAD, [^{14}C-Ado]-NAD and [^{3}H-NMN]-NAD, one double-labeling experiment was started by mixing tritium-labeled (pyridine-near ribose) and carbon-labeled (purine-near ribose) $NAD^{\oplus}$. In order to distinguish between either end group, pyridine-near ribose (C-1″) or purine-near ribose (C-2′ or C-3′), respectively, as the link toward protein, the incubated nuclei were first treated with phosphodiesterase followed by neutral hydroxylamine. From the structure of oligo-ADPR, the following labeling patterns would be expected:

Type of ADPR (chain-end)-protein linkage	C-1″		C-2′ or C-3′	
labeled NAD used	(^{3}H-ribose)	(^{14}C-ribose)	(^{3}H-ribose)	(^{14}C-ribose)
Solubilized by phosphodiesterase				
AMP	unlabeled	labeled	absent	absent
iso-ADP-ribose	labeled	labeled	labeled	labeled
ribose-5-phosphate	absent	absent	labeled	unlabeled
Subsequently solubilized by hydroxylamine				
AMP	absent	absent	unlabeled	labeled
ribose-5-phosphate	labeled	unlabeled	absent	absent

Table 1. Radioactive fractions isolated from ADP-ribosylation experiments of nuclear proteins (data as dpm eluted from Dowex 1×8).

Labeled NAD used	Double labeling*) with		Single labeling*) with				
	^{3}H-Ribose	^{14}C-Ribose	^{3}H-Ribose		^{14}C-Ribose		^{14}C-adenine
PDE	exp. 1	exp. 1	exp. 2		exp. 3		exp. 4
H_2O	26,995	5,576	1,083	14,784	2,992	1,129	8,650
0.1 M HCOOH	8,557	6,044	6,280	14,049	3,411	15,328	11,017
0.25 M HCOOH (AMP)	2,934	34,797	–	454	57,989	58,882	110,759
6 M HCOOH (ADPR, iso-ADPR, R-5-P)	53,051	33,957	45,266	41,841	49,194	48,697	165,506
6 M HCOOH + 0.4 M $HCOONH_4$ (oligo ADPR)	4,029	1,432	750	500	1,379	12,487	106,386
NH_2OH							
H_2O	397	–		367		112	1,618
0.1 M HCOOH	–	–		900		–	–
0.25 M HCOOH (AMP)	–	15,920		–		17,921	47,356
6 M HCOOH (ADPR, iso-ADPR, R-5-P)	98,747	40,845		22,180		19,418	113,384
6 M HCOOH + 0.4 M $HCOONH_4$ (oligo ADPR)	35,044	21,068		4,038		5,611	45,002
dpm's applied on Dowex	88,468	83,026	64,433	68,734	136,279	124,565	420,154 PDE
dpm's applied on Dowex	130,396	84,987		32,793		49,556	251,250 NH_2OH

*) ^{3}H-ribose resides in the NMN moiety, ^{14}C-ribose in the AMP moiety, respectively, of the NAD used for incubation.

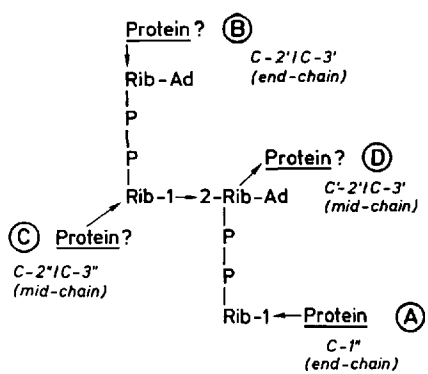
When the corresponding fractions were analyzed, the results were as follows in Table 1.

Recovery of radioactivity was in the average −2 % in 7 experiments with PDE (max. +10 %, min. −16 %) and in the average −8 % in 5 experiments with NH_2OH (max. +3 %, min. −13 %).

The pattern of the phosphodiesterase-released products is compatible with a C-1″ link, i.e. pyridine-near (A in the scheme below): However, subsequent treatment with hydroxylamine again liberates AMP, much iso-ADP-ribose is found, and ribose-5-phosphate cannot be detected with sufficient certainty. In these experiments, NaOH instead of NH_2OH did not lead to grossly different results.

If, however, hydroxylamine is employed without foregoing phosphodiesterase action (data not shown), highly labeled AMP is found (see also (21)) which became solubilized by NH_2OH. Such a reaction might possibly stem from some slight phosphodiesterase or ADP-ribose glycohydrolase action during the incubation of nuclei, but it certainly requires C-2′ or C-3′ (purine-near) in chain-end position as protein link (B in the scheme below). These results are thus compatible with an equivocal situation in that either ribose of $NAD^{\oplus}$ may be engaged in bonding to nuclear proteins in vitro (22).

However, the occurrence of labeled iso-ADP-ribose when hydroxylamine was employed after phosphodiesterase cannot be explained by either chain-end link to nuclear proteins but seems to require a different type of covalent bond as follows:



Therefore either C-2″ and/or C-3″ (C), or respectively C-2′ and/or C-3′ (D) in mid-chain position are suggested in addition to the end-chain positions C-1″ and, respectively, C-2′ and/or C-3′. Thus phosphodiesterase would gain additional cleavage sites once the protein link has been broken by hydroxylamine. The prerequisite that phosphodiesterase action was exhaustive prior to hydroxylamine incubation has regularly been checked and was always met (see also under Methods).

These data are valid for the NH_2OH-sensitive conjugates between protein and ADP-ribose formed in vitro; it follows from their interpretation

that the attachment sites both at nuclear proteins and at ADP-ribose moieties may be several-fold. In addition, however, mechanisms and sequence of oligo- and poly-ADP-ribose formation would need a thorough revision if C-2′/C-3′ and/or C-2″/C-3″ act as additional connecting points towards nuclear proteins. Data of Miwa et al. (23) on cross-linked oligo-ADP-ribose, of Gill (24) on ADP-ribose prolongation, of Tanaka (25) on oligo-ADP-ribose bonds to protein, of Suhadolnik (26) with 3′dNAD and 2′dNAD, as well as of Stone et al. (27) on histone H1-poly-ADP-ribose dimer molecules are, to say the least, not in contradiction to the reported findings and thus support the suggestion of this paper, to consider again, in conjunction of the multifunctional aspects of (6) protein ADP-ribosylation, the chemical aspects of the covalent bonds involved.

The data reported above are obtained in vitro and may not necessarily apply to in-vivo situations. Since, however, feeding of nicotinamide (28) or its intraperitoneal administration (2) enhances ADP-ribosylation of proteins, in-vivo effects of high doses of nicotinamide are established and lead to the question if the data reported above are of significance for megatherapy attempts with niacin. It is the suggestion of the present authors that the demonstrated multiplicity of protein conjugates with ADP-ribose, whose consequences cannot be overlooked at the time being, should rather restrict the use of niacin in doses which exceed by far nutritional requirements.

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