QUANTITATION OF HYDROXYLAMINE SENSITIVE MONO(ADENOSINE DIPHOSPHATE RIBOSE) RESIDUES IN DIFFERENT HEPATIC TISSUES

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1. Introduction

ADP-ribosylation of nuclear proteins in vitro was first shown by Hayaishi's group [1], subsequent work from Dietrich et al. [2] placing special emphasis on the existence of mono(ADPR) residues rather than poly(ADPR) in the protein conjugates. However, secondary degradation of oligo- or poly (ADPR) residues to the monomer has not been ruled out in these experiments. In vitro experiments using short-term incubation of nuclei followed by immediate inactivation of enzymes revealed considerable differences in tissues with widely differing proliferation rates, in the levels of monoADP-ribosylated nuclear proteins susceptible to NH_2OH [3]. We were interested in finding out whether this in vitro correlation reflected the in vivo situation. Since no method was available for the quantitation of mono-(ADPR) residues we have developed an isotope dilution procedure for measuring NH_2OH-sensitive, protein-bound mono(ADPR), and in this communication report on the endogenous levels of such ADPR residues in different tissues. The data show rather low concentrations of mono(ADPR) residues in vivo, reaching only 1–5% of the level of its precursor NAD. Proliferating tissues have significantly lower monoADP-ribosylated proteins than adult rat liver.

2. Materials and methods

2.1. *Preparation of acid-insoluble tissue fractions*

Freeze-clamped rat (♂, Wistar) livers (60 g adult; 25 g 1–2 days old neonatal) were homogenized with 300 ml (150 ml for neonatal) 10% TCA, washed twice with 10% TCA, 4 times with 96% ethanol and twice with ether. Zajdela Hepatoma Cells* were propagated and freed from erythrocytes as described previously [4]. 28 g (2.8×10^9 cells) were homogenized with 150 ml 10% TCA and treated as described above.

2.2. *Determination of mono(ADPR) residues*

NH_2OH extraction: The ether dried preparations were resuspended in 0.5 M NH_2OH pH 7.5 (50 ml for the adult preparation, 25 ml for the neonatal and hepatoma preparations) and incubated with [^{3}H]-ADPR (227 nmol, 1×10^7 dpm) for 1 hour at 37°C. 50% TCA was added to give a final concentration of 10%, the preparation centrifuged, washed twice with 10% TCA, the supernatants being kept each time. The pellet was then washed with 96% ethanol (2 ×) and ether (2 ×), and the dry preparation again extracted with NH_2OH and TCA as described above. The resulting three TCA supernatants were combined with the first TCA supernatants, extracted with water saturated ether (4 ×) and concentrated by rotary evaporation. Desalting of the NH_2OH extracts was achieved by chromatography on Sephadex G-10 columns (100 × 5 cm, flow rate = 100 ml/hr). The fractions containing radioactivity were pooled and concentrated to about 5 ml by rotary evaporation. Alkaline phosphatase digestion of the desalted

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preparation was done in the presence of 5 mM Mg acetate, 100 mM Tris acetate pH 7.5 and 21 units alkaline phosphatase (300 U/mg, E. Merck, Darmstadt) in a total volume of 7 ml (90 min 37°C). Anion exchange chromatography of the reaction mixture was performed after adjustion to pH 4.0 (1 N HCl) on a Dowex 50-X4 (200–400 mesh, H^+ form) column (2.5 × 30 cm; elution with H_2O; 60 ml/hr). The fractions containing radioactivity were pooled, the pH adjusted to pH 5.0 with 1 N KOH, and concentrated to 200 μl. The preparation was then purified by paper chromatography, first in the isobutyric acid/NH_3 system [5] (after a pre-run in 80% ethanol) three times, and then twice in the tetrahydrofurfurol system [6]. For every chromatographic run, appropriate paper blanks were also analyzed for A_{260} and the value substracted.

Conversion of the ADPR to AMP: adenosine and inosine and subsequent paper chromatography was performed with the concentrated paper eluates. About 200 μl were incubated (90 min at 37 °C) with 200 mM Tris–HCl pH 8.0 and 15.2 mU phosphodiesterase II (phosphatase free, 0.76 U/mg, E. Merck, Darmstadt) in a total volume of 500 μl. The reaction mixture was purified by paper chromatography in the isobutyric acid/NH_3 system, after a 6-hr pre-run in 80% ethanol, and the eluted material was concentrated. The resulting 200 μl were incubated with 100 mM Tris acetate pH 7.5, 5 mM Mg acetate and 1.5 U alkaline phosphatase (*E. coli,* 300 U/mg, Boehringer, Mannheim) in a final volume of 500 μl (90 min at 37 °C). The reaction mixture was purified by paper chromatography in 80% ethanol and the eluted preparation was concentrated. 200 μl were incubated with 100 mM Tris acetate pH 7.4 and 5 U adenosine deaminase (200 U/mg, Boehringer, Mannheim) in a total volume of 500 μl (90 min at 37 °C). The reaction mixture was purified by paper chromatography (after a pre-run in 80% ethanol) in either water and/or the isobutyric acid/NH_3 system [5].

The specific radioactivity after each purification step was determined by measuring A_{260} and dpm.

DNA was determined according to Burton [8], NAD according to Caiger et al. [9].

[^{3}H]NAD was synthesized from NMN and [^{3}H]-ATP as previously described [7]. [^{3}H]ADPR was prepared from [^{3}H]NAD by digestion with NADase (calf spleen, Boehringer, Mannheim). The [^{3}H]ADPR obtained was purified by paper chromatography in the isobutyric acid/NH_3 system [5].

3. Results and discussion

3.1. *Isotope dilution procedure for the quantitation of protein-linked NH_2OH-sensitive mono(ADPR) residues*

The liberation of nuclear [^{3}H] $(ADPR)_n$ residues into an acid-soluble form by KOH or NH_2OH treatment of nuclei incubated with adenine-labeled NAD was first reported by Hayaishi's group [1]. Part of these released $(ADPR)_n$ residues is in the form of mono(ADPR) as shown by chromatographic analysis [2,3]. Based on the NH_2OH-induced release of acid-soluble $(ADPR)_n$, an isotope dilution method was developed which allowed quantitation of the mono(ADPR) residues, independent of losses during the many purification steps necessary to obtain constant specific radioactivity.

The purification scheme consisted of precipitation of freeze-clamped livers by cold trichloroacetic acid, liberation of protein-bound $(ADPR)_n$ residues by 0.5 M NH_2OH in the presence of a defined amount of [^{3}H]ADPR and reprecipitation of all acid-insoluble material in this extract by trichloroacetic acid. The acid-soluble, desalted fraction was treated with alkaline phosphatase to convert contaminating mononucleotides to nucleosides, and subjected to Dowex-50 chromatography to retain the nucleosides. Final purification of ADPR was achieved by repeated paper chromatography in different systems, each to constant specific radioactivity, and subsequent enzymic conversion to specific derivatives. Besides other contaminating nucleotides, dimers, trimers and higher oligomers of ADPR are also clearly eliminated by paper chromatography (Stone et al., unpublished data). The overall purification resulted in a constant specific radioactivity of ADPR and its derivatives in several systems (table 1).

The endogenous concentrations of NH_2OH-sensitive protein bound mono(ADPR) residues were calculated according to the following equation:

$$\text{nmol endogenous ADPR/mg DNA} = \left[\frac{\text{spec. act. of exog. ADPR}}{\text{spec. act. of purified ADPR}} - 1\right] \times \text{nmol exog. ADPR/mg DNA}$$

Table 1
Purification of ADPR and its derivatives to constant specific radioactivity (neonatal rat liver)

Purification step	Specific radioactivity (dpm × 10^{-4}/nmol)
NH_2OH extraction	0.0041
G-10 chromatography	0.0096
Dowex-50 chromatography	0.16
1st Isobutyric acid chromatography	0.55
2nd Isobutyric acid chromatography	0.55
3rd Isobutyric acid chromatography	0.57
1st Tetrahydrofurfurol chromatography	2.06
2nd Tetrahydrofurfurol chromatography	2.21
Conversion to AMP	2.27
Conversion to adenosine	2.12
Conversion to inosine	2.19

Besides the specific radioactivities of the [^{3}H]-ADPR added, and of the purified ADPR (or its derivatives), the amount of [^{3}H]ADPR added to a defined amount of tissue extract as well as the DNA content of the tissue must be known.

Applied to the data obtained with adult rat liver, a mean value of 5.28 ± 0.22 nmol mono(ADPR) residues/mg DNA was found from two separate analyses (cf. table 2). This level is very similar to the concentration of poly(ADPR) residues found in the same tissue [10]. When compared to the concentration of NAD (cf. table 2), an unusual relationship is seen: The level of the reaction product in vivo is 20–70 times *lower* than the level of the substrate NAD.

3.2. *Comparison of endogenous mono(ADPR) levels in tissues with different proliferation rates*

Poly(ADPR) has been implicated in the regulation of cell proliferation and DNA synthesis [cf. 11], although no correlation of synthetase and degrading activities with proliferation rates in different liver tissues was found [4,12]. In contrast to the somewhat contradictory observations with respect to poly(ADPR), considerable quantitative differences of in vitro mono(ADP)-ribosylated nuclear proteins in tissues with different proliferation rates were seen [3].

When the in vivo levels of mono(ADPR) residues released by NH_2OH were determined in various hepatic tissues, a certain dependency on the

Table 2
Mono(ADPR) residues and NAD content of various hepatic tissues

Tissue	mono(ADPR) residues (nmol/mg DNA)	NAD content (nmol/mg DNA ± SEM)
Adult	5.28	359 ± 14
Neonatal	2.19	81 ± 1
Hepatoma	2.11	42 ± 2

Mono(ADPR) and NAD were determined as described in Materials and methods. For the mono(ADPR) determinations the results represent the values obtained from the pooled livers of 8 adult rats, 80 neonatal rats and the hepatoma cells from 21 rats. The reproducibility was checked in two determinations of adult livers and a value of 5.28 ± 0.22 (SEM) obtained. Each NAD content determination represents the means from 3 separate rats.

proliferation rate was also seen (table 2): While adult rat liver (low proliferation) had the highest level, neonatal liver and Zajdela hepatoma (high proliferation) exhibited lower concentrations of mono-(ADP)-ribosylated proteins. The increasing proliferation rate from adult over neonatal liver to the fast growing Zajdela hepatoma is paralleled by a corresponding increase in [^{3}H]thymidine incorporation into DNA [4], and a decrease in NAD concentration (table 2). NAD levels have been shown to exhibit an inverse relationship to cell proliferating rate [13–15]. Although both proliferating tissues, neonatal liver and Zajdela hepatoma, showed definite lower levels of mono(ADPR) residues than adult liver, the values do not correlate exactly with the proliferation rate as do the NAD levels. Consequently, there seems to be no direct correlation between NAD levels and the amount of mono(ADP)-ribosylated proteins indicating that cellular NAD concentrations per se do not determine the rate of ADP-ribosylation. The high ratio of NAD to mono(ADPR) residues also favors such an interpretation.

It should be ponted out, that the NH_2OH-sensitive mono(ADPR) residues measured here do not represent the total mono(ADP)-ribosylated proteins. Recently, Adamietz and Hilz have shown by quantitative analysis, that at least two types of bonds occur in $(ADPR)_n$-protein complexes, one comprising the bulk of the $(ADPR)_n$ residues and being susceptible to NH_2OH as well as to alkali, the other being NH_2OH-resistant [16]. So far, no test for the quantitation of the NH_2OH-resistant fraction has been developed.

The findings that the acid-soluble mono(ADPR) in the NH_2OH extract must be purified by a factor of 500 to obtain pure ADPR (table 1) clearly shows that most of the A_{260}-absorbing material in the extract is *not* identical with (ADPR) residues, thus limiting the significance of in vivo labeling experiments where extensive purification of the NH_2OH extract has not been performed [cf. 17].

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References

[1] Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M. and Hayaishi, O. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 781–786.
[2] Dietrich, L. S. and Siebert, G. (1973) Hoppe-Seyler's. Z. Physiol. Chem. 354, 1133–1140.
[3] Adamietz, P., Bredehorst, R., Oldekop, M. and Hilz, H. (1974) FEBS Lett. 43, 318–322.
[4] Hilz, H. and Kittler, M. (1971) Hoppe-Seyler's. Z. Physiol. Chem. 352, 1693–1704.
[5] Shima, T., Hasegawa, S., Fujimura, S., Matsubara, H. and Sugimura, T. (1969) J. Biol. Chem. 244, 6632–6635.
[6] Carpenter, D. S. (1952) Analyt. Chem. 24, 1203–1204.
[7] Nolde, S. and Hilz, H. (1972) Hoppe-Seyler's. Z. Physiol. Chem. 353, 505–513.
[8] Burton, K. (1968) Methods Enzymol. XII, 163–166.
[9] Caiger, P., Morton, R. K., Filsell, O. H. and Harrett, I. G. (1962) Biochem. J. 85, 351–359.
[10] Stone, P., Lengyel, H., Kittler, M. and Hilz, H., unpublished experiments.
[11] Sugimura, T. (1973) in: Prog. Nucleic Acids and Mol. Biol. (Davidson, J. N. and Cohn, W. E., eds.) Vol. 33 pp. 127–151, Academic Press, Inc., New York.
[12] Leiber, U., Kittler, M. and Hilz, H. (1973) Hoppe-Seyler's. Z. Physiol. Chem. 354, 1347–1350.
[13] Jedeiking, L. A. and Weinhouse, S. (1955) J. Biol. Chem. 213, 271–280.
[14] Ferris, G. M. and Clark, J. B. (1971) Biochem. J. 121, 655–662.
[15] Jacobson, E. L., Jacobson, M. K. and Bernofsky, C. (1974) FEBS Lett. 47, 23–25.
[16] Adamietz, P. and Hilz, H. (1975), submitted for publication.
[17] Dietrich, L. S., Haus, J. and Siebert, G. (1973) FEBS Lett. 37, 228–230.