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ADP-RIBOSYLATION OF HUMAN SERUM PROTEINS PROMOTED BY ENDOGENOUS NAD GLYCOHYDROLASE ACTIVITY

R.Nurten[1], İ.Üstündağ[1], N.Sayhan[1] and E.Bermek[1,2]

[1] Department of Biophysics
I.U. Istanbul Faculty of Medicine
34390 Çapa-İstanbul, Turkey

[2] Marmara Research Center
41470 Gebze-Kocaeli, Turkey

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Incubation of human serum samples with [adenine-^{14}C]NAD resulted in a time- and dose-dependent incorporation of adenine moiety into CCl_3COOH-precipitable material. Incorporated radioactivity was relatively resistant to neutral hydroxylamine, but was completely released by treatment with NaOH. An incorporation was observed also after preincubation of NAD with NAD glycohydrolase from pig brain.
NAD glycohydrolase activity in serum samples was then shown spectroscopically in an assay coupled to alcohol oxidation. Thus, this reaction was implicated to be due to the binding of ADP-ribose, formed under the action of a soluble, endogenous NAD glycohydrolase activity, to serum proteins. Analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis (PAGE) and autoradiography indicated that a polypeptide of 97 kD, but also two further polypeptides of higher molecular weight and serum albumin, were labelled after incubation with radioactive NAD.

Adenosine diphosphate(ADP)-ribosylation of proteins is a post-translational modification reaction involving the transfer of ADP-ribose groups from NAD to acceptor proteins (see for recent reviews references 1,2). ADP-ribosylations are classified as mono ADP-ribosylation and poly ADP-ribosylation, depending upon the length of the transferred group.

Mono ADP-ribosylation has been originally found as a reaction catalyzed by certain bacterial toxins (1,2). These, acting as specific ADP-ribosyltransferases, modify acceptor proteins involved in some key cellular processes. Toxin-catalyzed ADP-ribosylation reactions are, in general, well characterized in respect to their mechanistics,

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acceptor sites of ADP-ribose groups as well as physiological effects (1,2). Work of recent years has additionally revealed the presence of endogenous mono ADP-ribosylations in a number of different systems (3-8). In contrast to that of bacterial toxins, the physiological significance of the endogenous mono ADP-ribosylation reactions remains yet to be established. Moreover, free ADP-ribose formed under action of endogenous NAD-glycohydrolase(s) can also bind to proteins, simulating ADP-ribosyltransferase activity (9,10).

Here, we report, as a contribution to the latter issue, the presence of an apparant NAD glycohydrolase activity in serum which gives rise to the formation of ADP-ribose-serum protein conjugates via free ADP-ribose.

MATERIALS AND METHODS

[adenine-^{14}C(U)]NAD, specific activity 518 Ci/mol, and [adenylate-^{32}P]NAD, specific activity 30 Ci/mmol, were purchased from DuPont (NEN). Snake venom phosphodiesterase and alcohol dehydrogenase were products of Boehringer (Mannheim). NAD glycohydrolase from pig brain and all chemicals of analytical grade were from the Sigma Chemical Company. Serum samples were obtained from young, healthy individuals. Care was paid that samples were free of hemolysis. If not otherwise indicated, serum samples were subjected prior use to overnight dialysis against 50 mM Tris-HCl, pH 7.4, 7 mM 2-mercaptoethanol, 0.1 mM EDTA and 250 mM sucrose (=dialysis buffer). The samples kept in frozen state appeared to preserve their activity in ADP-ribosylation for some weeks. Reactions in serum samples were carried out in the presence of 10 μM[^{14}C]NAD for two hours at 37°C. The reaction medium was adjusted so that it contained 8 mM $CaCl_2$ in dialysis buffer. After incubation, CCl_3COOH-precipitable radioactivity was determined as described (11). NaDodSO$_4$/PAGE (12) of serum samples were performed after incubation with [^{14}C]NAD as above or with 2μCi [^{32}P]NAD. Bands of ^{32}P labelled proteins were determined by autoradiography on Coomasie blue stained, destained and dried gel slabs exposed to Kodak X-Omat K films. ^{14}C labelled protein(s) were, in turn, determined by cutting out the regions of the gel slabs corresponding to protein bands and by subsequent incubation of the gel slices for 24 h at 50° C in 0.5 ml 30% H_2O_2 in liquid scintillation vials. The samples were finally counted in Bray's solution in liquid scintillation spectrometer.

NAD glycohydrolase activity (in serum samples) was assayed by coupling to alcohol oxidation in the presence of an excess of alcohol dehydrogenase (13). The procedure as described by the producer company (Sigma Chemical Company) was hereby followed. For analysis of ADP-ribose adducts, serum samples incubated with radioactive NAD, were centrifuged in Amicon MPS1 filter holders for 15 min at 2000xg and retentates dialysed against 20 mM $(NH_4)HCO_3$. They were digested with snake venom phosphodiesterase for 90 min at 37° C. The samples were then centrifuged again and the ultrafiltrates subjected to chromatographic analysis on polyethyleneimine (PEI)-

cellulose thin layers (14). The chromatography was developed using 0.3 M lithium chloride as solvent and AMP, ADP-ribose, NAD and adenosine as markers. Following chromatography, tracks on PEI-cellulose thin layer plates (Schleicher and Schüll, Dassel) were divided in 1 cmx0.5 cm areas and PEI-cellulose in these areas was scraped into vials and analysed for radioactivity in a liquid scintillation counter. Elongation factor 2(EF-2) from rat liver, ADP-ribosylated endogenously (11) and submitted to similar treatment as serum samples, was used as reference.

RESULTS

When human serum was incubated with [adenine-^{14}C]NAD, there was an incorporation of radioactivity into CCl_3COOH-precipitable material. The reaction was time-and dose-dependent, but, being slow, it reached its maximum value in about two hours (Fig.1). Prior dialysis of the serum samples had a slightly enhancing effect. Moreover, the addition of divalent ions (Ca^{2+}(8mM) or Mg^{2+}(10 mM)) stimulated the reaction upto onefold. Incorporated radioactivity was in form of monomeric ADP-ribose units, as indicated by PEI-cellulose chromatography of snake venome phosphodiesterase digest of CCl_3COOH-precipitable reaction products (14) (not shown).

Incorporated radioactivity was released only partially after a 10 hour incubation in the presence of NH_2OH; treatment with NaOH resulted, in turn, in its complete release (Table 1). In the light of the previous reports (9,10), the presence of NH_2OH-resistant mono ADP-ribose-protein conjugates implicated that the observed

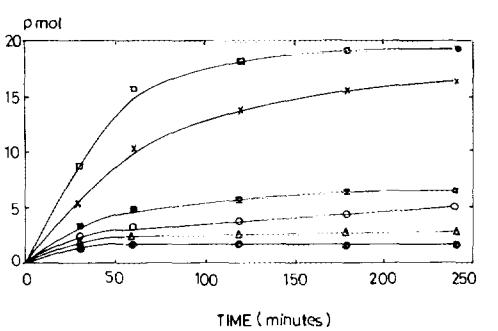

Fig.1. Dependence of the rate and extent of ADP-ribosylation in serum upon NAD concentrations. Reactions were carried out in 100 µl dialyzed serum samples and NAD varied as indicated. Experimental conditions were otherwise as described under Materials and Methods. 15 µl aliquots taken at the indicated periods were assayed for incorporated radioactivity (11). NAD concentrations: (●-●), 1.5 µM; (Δ-Δ), 3.0 µM; (o-o), 5.0 µM; (■-■), 7.5 µM; (x-x), 10.0 µM; (□-□), 12.5 µM.

Table 1. Stability of ADP-ribosyl conjugates in serum in the presence of hydroxylamine or sodium hydroxide

Addition	CCl_3COOH-precipitable ADP-ribose (pmol)	
	1 h	10 h
None (control)	8.7	8.6
Hydroxylamine (0.1 M)	5.9	5.9
Hydroxylamine (0.5 M)	5.3	5.2
NaOH (0.1 M)	1.9	0.7
NaOH (0.5 M)	1.5	0.4

The experimental conditions were as described under Materials and Methods. [^{14}C]ADP-ribosylated serum samples were incubated in the presence of hydroxylamine or NaOH for 1 or 10 hours at 37°C. Thereafter, CCl_3COOH-precipitable radioactivity was determined as described (11).

incorporation may be due to a nonenzymic binding of ADP-ribose formed under the action of an NAD glycohydrolase activity in serum. That the nonenzymic binding of ADP-ribose can simulate an apparant ADP-ribosylation of serum proteins is shown in Table 2. Radioactive NAD after preincubation with NAD glycohydrolase from pig brain gave rise similarly to an incorporation of radioactivity into serum protein fraction. Unlabeled NAD as well as ADP-ribose inhibited both reactions by isotope dilution. Heat treatment of serum, in turn, abolished them. Nicotinamide, a product of NAD hydrolase and glycohydrolase reactions, and, to lesser degree, isonicotinic acid hydrazide (INH), an inhibitor of NAD glycohydrolase, were also inhibitory. The incorporation from both NAD and ADP-ribose revealed in a similar manner two pH optima at 4(-5) and 9 (Fig.2).

NAD glycohydrolase activity in serum was finally demonstrated spectroscopically in an assay coupled to alcohol oxidation in the presence of a saturating amount of alcohol dehydrogenase. As indicated in Table 3, the incubation of NAD with serum resulted in the inhibition of the subsequent alcohol oxidation reaction, due to NAD hydrolysis. Nicotinamide and INH inhibited again to varying degrees the serum specific NAD glycohydrolase activity.

Table 2. Effect of different compounds and treatments upon the incorporation of radioactivity from NAD versus ADP-ribose into serum proteins

Treatment and additions	ADP-ribose incorporated (pmol)
NAD	
None	27.0
+NAD (unlabeled), 100 μM	9.1
+ADP-ribose (unlabeled), 100 μM	9.6
+Nicotinamide, 1 mM	9.0
+INH, 8 mM	18.0
+Serum (5 min 50°C)	1.5
ADP-ribose	
None	30.8
+NAD (unlabeled), 100 μM	5.5
+ADP-ribose (unlabeled), 100 μM	6.1
+Nicotinamide, 1 mM	8.2
+INH, 8 mM	16.2
+Serum (5 min 50°C)	1.3

The experimental conditions were as indicated under Materials and Methods. 30 μl reaction mixtures contained 20 μl dialyzed serum. 25 μl aliquots were plated on GF/A glass fibers and CCl_3OOH-precipitable radioactivity was determined as described (11). ADP-ribose was prepared by incubation of NAD(1 mM) in the presence of 1 mg/ml of particulate NAD glycohydrolase from pig brain for 15 min at 25°C. In case radioactive NAD was used, these values were reduced fivefold. Following incubation, the reaction mixture was centrifuged and supernatant used as ADP-ribose.

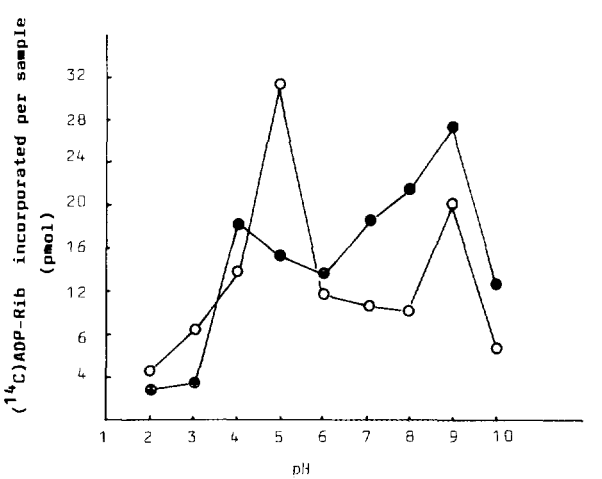

Fig.2. pH dependence of ADP-ribosylation in serum. Reactions were carried out in the presence of either NAD (●-●) or ADP-ribose (o-o) and reaction pH was varied as indicated. The buffer systems used (20 mM each) were acetate buffer for the range pH 4-5, phosphate buffer for pH 6-7, Tris-buffer for 7-9 and bicarbonate buffer for 9-10.

Table 3. NAD glycohydrolase activity in serum

System	NAD glycohydrolase (units.min^{-1}.ml^{-1})
Control	
+NAD glycohydrolase	0.16
+NAD glycohydrolase, +nicotinamide	0.09
+NAD glycohydrolase, +INH	0.07
+serum	0.14
+serum, +nicotinamide	0.11
+serum, +INH	0.08

NAD glycohydrolase activity was determined as described under Materials and Methods. The amount of NAD hydrolyzed in the first reaction was determined by measuring the residual amount of NAD, using the second reaction based on alcohol dehydrogenase catalyzed alcohol oxydation. NAD consumed was hereby calculated from A_{340} difference between the control and NAD glycohydrolase containing samples. One unit is defined as 1 micromole of NAD hydrolyzed to nicotinamide and ADP-ribose per minute at pH 7.3 at 37°C. NAD glycohydrolase (from pig brain, Sigma) concentration was adjusted to 0.2 unit. ml^{-1}. End concentrations of INH and nicotinamide were 8 mM and 1 mM, respectively. First and second incubations were each carried out for 15 minutes.

Finally, the acceptor molecule(s) of the ADP-ribosylation reaction were investigated by NaDodSO$_4$/polyacrylamide gel electrophoresis of the serum samples following incubation with either [^{14}C]NAD or [^{32}P]NAD. As shown in Fig.3A, a polypeptide of 97 kD was predominantly labelled after incubation with [^{14}C]NAD. However, upon incubation with [^{32}P]NAD, two further polypeptides of higher molecular weight as well as serum albumin were found to be additionally labelled (Fig.3B).

DISCUSSION

The results of this investigation indicate the presence of an NAD glucohydrolyse activity in serum. ADP-ribose released as consequence of this activity appears to form hydroxylamine-resistant conjugates with the serum protein fraction. Free ADP-ribose is known to form Schiff bases with amino groups of proteins (10). Hydrolysis of NAD by NAD glycohydrolases gives rise to nonenzymic ADP-ribosylation of specific acceptors in mitochondria (9). This reaction sequence which simulates ADP-ribosyltransferase activity may, in part, account for hydroxylamine-resistant ADP-ribose-protein conjugates of intact tissues (9). NAD glycohydrolase activity in serum shows resemblence with the

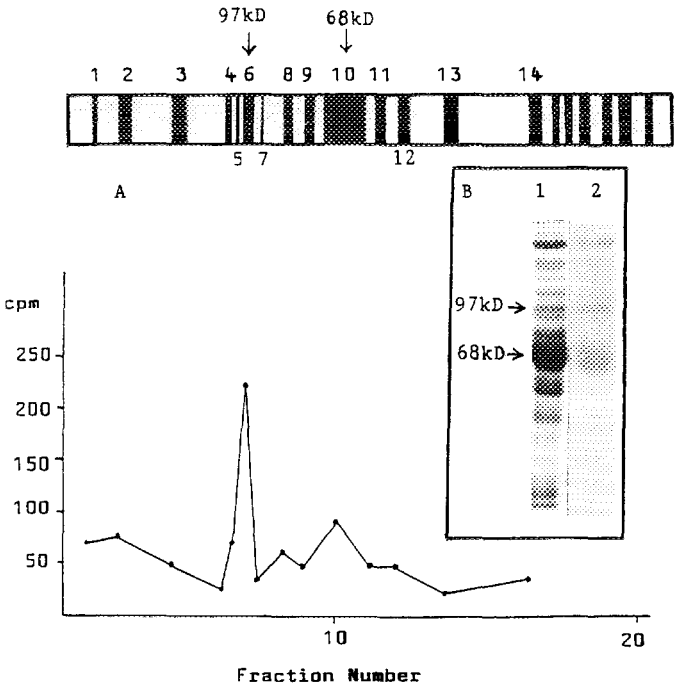

Fig.3. A) Analysis of ADP-ribosylated serum proteins by NaDodSO$_4$/PAGE, following incubation with [^{14}C]NAD. B) Analysis of ADP-ribosylated serum proteins by NaDodSO$_4$/PAGE (1) and autoradiography (2), after incubation with [^{32}P]NAD. Experimental conditions were as described under Materials and Methods. Phosphorylase b and bovine serum albumin as well as catalase and ovalbumin (not shown) were used as markers.

previously described activity in mitochondria and submitochondrial particles from beef heart and rat liver, in regards to pH optima and to requirements for bivalent cations (9). The sensitivity to inhibition by INH was not as marked as reported (9). The differences in INH sensitivity of NAD glycohydrolases from different mammals account likely for this variance (15).

The question of the physiological significance of this activity is presently unknown. The data to be reported elsewhere indicate, however, its enhancement in several neoplastic cases. Thus, it appears to be of importance to investigate the correlation of the observed activity in serum with conditions of active cell proliferation and/or with anti-tumor human therapy. Induction of DNA repair by alkylating agents is known to lead to stimulation of poly(ADP-ribose) turnover (16-18) and a concomittant production of free ADP-ribose under the action of poly ADP-ribose glycohydrolase.

NAD glycohydrolase(s) are normally found in particulate form(s), i.e., associated with the microsomal fraction in the cell (15,19-21). A few percent of the NAD-glycohydrolase activity is, in turn, located in the nucleus, involved in poly ADP-ribose metabolism (1,16). Hence, the appearance of a soluble form of NAD glycohydrolase in serum deserves special attention. It is of interest to note that earlier reports have indicated the appearance of soluble NAD glycohydrolase in tuberculous guinea pigs accompanied by eighfold elevation of the related activity in plasma (22).

ADP-ribose appears to bind to only a restricted number of serum proteins, with preference to a polypeptide of 97 kD. Thus, a certain specificity is implicated in the observed binding. The abolishment upon heat treatment of serum underlines the importance of proper conformation of the acceptor proteins for the binding of ADP-ribose. Data to be reported elsewhere attests to the polypeptide of 97 kD as the sole acceptor in randomly choosen serum samples from a tumor marker laboratory. Moreover, this polypeptide reveals in some cases a particularly high intensity of label. The factors enhancing the reactivity of the acceptor protein(s) in the binding remain yet to be clarified.

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