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Evidence for an Inhibitory Effect Exerted by Yeast NMN Adenylyltransferase on Poly(ADP-ribose) Polymerase Activity

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ABSTRACT: We have previously reported for the first time the purification to homogeneity of the enzyme NMN adenylyltransferase (EC 2.7.7.1) from yeast and its major molecular and catalytic properties. The homogeneous enzyme was found to be a glycoprotein containing 2% carbohydrate and 1 mol of adenine residue and 2 mol of phosphate covalently bound per mole of protein. Such a stoichiometry, apparently consistent with that of ADP-ribose, prompted us to further investigate the possibility that NMN adenylyltransferase could be subjected to poly(ADP-ribosylation) in vitro in a reconstituted system. Poly(ADP-ribose) polymerase was purified to homogeneity from bull testis by means of a rapid procedure involving two batchwise steps on DNA–agarose and Reactive Blue 2 cross-linked agarose and a column affinity chromatography step on 3-aminobenzamide–Sepharose; the optimal conditions for the poly(ADP-ribosylation) of exogenous substrates were determined. When pure NMN adenylyltransferase was incubated in the presence of the homogeneous poly(ADP-ribose) polymerase, a marked inhibition of the polymerase was observed, both in the presence and in the absence of histones, while the activity of NMN adenylyltransferase was not affected. The inhibition could not be prevented by increasing the concentrations of either DNA or NAD. Mg^{2+} did not affect the activity or the inhibition. The significance of such a phenomenon is at present unknown, but it may be of biological relevance in view of the close topological and metabolic relationship between the two enzymes.

In addition to its universal function as a cofactor in oxidation–reduction reactions, NAD is known to be utilized as a substrate in ADP-ribosylation reactions. Quite uniquely the NAD synthesis from NMN and ATP is localized in the nucleus, catalyzed by the enzyme NMN adenylyltransferase (EC 2.7.7.1) strictly bound to chromatin (Siebert, 1963). The enzyme poly(ADP-ribose) polymerase (EC 2.4.2.30) catalyzes the polymerization of ADP-ribose moieties from NAD on target nuclear proteins and histones, as well as on the enzyme itself (automodification), resulting in NAD breakdown. Such a posttranslational modification has been related to many different nuclear events, such as differentiation, cell division, and DNA repair (Shall, 1985). Because of its ability to freely cross the nuclear envelope (Siebert, 1972), any depletion of NAD caused in the nucleus by the occurrence of events involving enhanced ADP-ribosylation is readily transferred as a signal to the cytoplasm, where NAD-related metabolic reactions can be accordingly regulated, and vice-versa (Loetscher et al., 1982). Despite the increasing interest in the physiological role of the poly(ADP-ribosylation) level, as well as in the identification of specific enzyme protein acceptors (Gaal & Pearson, 1985; Althaus & Richter, 1988), less interest has been focused on the other nuclear enzyme of NAD metabolism, NMNAT,[1] although it represents a key route for the synthesis of NAD within the cell.

On the basis of indirect evidence, a direct relationship between this enzyme and ADPRP has been suggested (Uhr & Smulson, 1982). To investigate its regulatory properties, we have purified for the first time to homogeneity the enzyme NMNAT from yeast (Natalini et al., 1986) and found evidence for its possible ADP-ribosylation; consistent with observations by others (Miwa et al., 1983; Mura, 1987), we also demonstrated in purified yeast nuclei the presence of an ADP-ribosylating activity, together with degrading activities both inactivating the ADPRP enzyme and hydrolyzing the

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[1] Abbreviations: NMNAT, NMN adenylyltransferase; ADPRP, poly(ADP-ribose) polymerase; ADPR, ADP-ribose; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 3-ABA, 3-aminobenzamide; 3-MBA, 3-methoxybenzamide; BSA, bovine serum albumin.

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ADP(ribose)–protein adducts (Ruggieri et al., 1988). The presence of such activities hampered further attempts to isolate oligo- or poly(ADP-ribosylated) forms of NMNAT and to purify the yeast ADPRP. The aim of this approach was twofold: to evaluate the possible existence of poly(ADPR) acceptor sites on NMNAT and to evidence some regulatory effect resulting from poly(ADP-ribosylation) in vitro. As an alternative approach, in the present work we purified the enzyme ADPRP from bull testis and used it as an ADP-ribosylating enzyme. Use of a heterologous reconstituted system has been reported for the poly(ADP-ribosylation) of wheat germ RNA-polymerase (Taniguchi et al., 1985) and of micrococcal nuclease (Tanaka et al., 1985) by means of bovine thymus ADPRP. This approach, therefore, may provide evidence, albeit not conclusive, of ADP-ribose acceptor sites on NMNAT and of any effect resulting from ADP-ribosylation. In this paper we describe both a rapid procedure for the purification of ADPRP from bull testis and an unexpected inhibitory effect of the homogeneous NMNAT preparation on the activity of the purified ADPRP in the reconstituted system.

Experimental Procedures

Materials

NMN, NAD, ATP, and histones (type IIA) were from Sigma. [*adenine*-2,8-^{3}H]NAD was from ICN Radiochemicals (21 Ci/mmol, 0.25 mCi/mL); [*adenylate*-^{32}P]NAD was from NEN (800 Ci/mmol, 5 mCi/mL). 3-Aminobenzamide and 3-methoxybenzamide were from Aldrich. Reactive Blue 2 cross-linked blue agarose resin was from Sigma. Enzymes and protein markers were from Sigma, Bio-Rad, or Boehringer Mannheim, as noted. Superose 12 HR10/30 FPLC column was from Pharmacia.

Methods

Assay for NMNAT Activity. Enzyme activity was assayed by a continuous spectrophotometric coupled enzyme assay (Ruggieri et al., 1988). The reaction mixture contained 60 mM HEPES buffer, pH 7.6, 1.18 mM NMN, 1.47 mM ATP, 20.7 mM $MgCl_2$, 35 mM semicarbazide hydrochloride, 0.45% (v/v) ethanol, 7.8 units of yeast alcohol dehydrogenase, 0.59 mg/mL bovine serum albumin, and an appropriate amount of sample to be assayed, in a final volume of 0.85 mL. The reaction was started by the addition of NMN and followed at 37 °C by the increase of absorbance at 340 nm, in a Varian DMS90 spectrophotometer.

Assay for ADPRP Activity. Radioactive assay was performed as previously described (Burthscher et al., 1986). The reaction mixture, in a 100 μL final volume, contained 80 mM Tris-HCl buffer, pH 8.0, 8 mM DTT, and 2 μg of activated DNA prepared as described (Loeb, 1969); histones (2 μg), either 10 μM [^{3}H]NAD or 10 μM [^{32}P]NAD, and 10 mM $MgCl_2$ were included in the reaction mixture as noted. The reaction was started by the addition of the appropriate amount of ADPRP and terminated, after 20 min at 25 °C, with the addition of an equal volume of 25% (w/v) TCA. After standing on ice for 10 min, the reaction mixture was filtered through a glass fiber filter (Whatman), followed by two washings with 5% TCA and two washings with a 1:1 mixture of ethanol/ether. The filter was dried in an oven at 60 °C for 10 min and then placed in a vial together with scintillation mixture and counted in a Beckman Model LS1801 scintillation counter. When the experiment was designed to measure the incorporation counts in individual electrophoretic bands following a reaction, the TCA precipitate was collected by centrifugation, washed as above, and then dissolved in electrophoresis sample buffer containing 2% SDS and run on 10% SDS–PAGE. The gel was stained as described below, dried over Whatman 3MM filter paper, and autoradiographed on Kodak X-Omat AR film.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis under denaturing conditions was conducted essentially as described (Laemmli, 1970). The sample was previously heated at 100 °C for 5 min in the presence of 2% SDS, 5% 2-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8. After electrophoresis, the gel was stained with the Bio-Rad silver staining kit.

Purification of NMNAT. The enzyme was purified from commercial yeast according to the previously reported procedure (Natalini et al., 1986). This preparation shows, upon native disc gel electrophoresis on 5% polyacrylamide gel, a single protein band (R_m = 0.4) corresponding to the enzyme band. Upon SDS–PAGE it runs as a single, M_r = 50 000, band. The native molecular weight was 200 000. Details of the procedure and criteria of homogeneity have been already reported by us (Natalini et al., 1986). It must be pointed out that, unlike the partially purified enzyme preparations from other sources (Ferro & Kuehl, 1975; Uhr & Smulson, 1982; Atkinson et al., 1964), the homogeneous yeast enzyme does enter the 5% polyacrylamide gel. For the purpose of the present investigation, the homogeneous NMNAT preparation was extensively dialyzed against 80 mM Tris-HCl, pH 8, and 10% glycerol to match with the ADPRP reaction mixture.

Purification of ADPRP. The enzyme was purified from bull testis with a rapid procedure consisting of two batchwise steps and a column affinity chromatography step on 3-ABA–Sepharose. The following procedure was carried out at 4 °C. Two hundred grams of bull testis tissue was homogenized in a Waring Blendor in an equal volume (v/w) of the following buffer: 100 mM Tris-HCl, pH 8, 1 mM EDTA, 12 mM 2-mercaptoethanol, 15% glycerol, 25 mM potassium metabisulfite (buffer A), containing 0.3 M NaCl and 0.3 mM PMSF. The homogenate was centrifuged at 9000*g* for 20 min and the supernatant (400 mL) mixed with 200 mL of DNA–agarose suspension prepared as described (Schaller et al., 1972) equilibrated with buffer A containing 0.2 M NaCl and stirred for 60 min at 4 °C. The suspension was centrifuged at 3000*g* for 5 min, the supernatant was discarded, and the resin was washed twice (600 mL) with the same equilibration buffer followed by centrifugation as above. The enzyme activity was eluted by incubating the resin with buffer A made 0.8 M NaCl, for 20 min at 4 °C, and centrifuging at 4000*g* for 5 min. The final volume of the resulting active pool was 300 mL (DNA–agarose fraction). This pool was diluted 4-fold with buffer A and mixed with 100 mL of packed Reactive Blue 2 cross-linked agarose resin, previously equilibrated with buffer A containing 0.2 M NaCl. The suspension was stirred for 30 min and then vacuum-filtered through a sintered-glass filter funnel at 4 °C. The flow-through was discarded, and the resin was washed with 5 volumes of buffer A containing 0.2 M NaCl, 2 mM ATP, and 2 mM AMP. The last wash was performed with the equilibration buffer until the optical density at 260 nm reached zero. The resin was then poured in a (5 × 10 cm) chromatography column. For the elution, buffer A was adjusted to 0.5 M NaCl, and 300 mL was used to elute the enzyme activity, collecting 10-mL fractions. The active fractions were combined in a pool (150 mL) (Blue–agarose fraction). This pool was diluted to a 0.3 M NaCl concentration and loaded onto a 3-ABA–Sepharose resin (Burthscher et al., 1986) equilibrated with buffer A containing 0.3 M NaCl. The resin was then packed in a (1.0 × 5 cm) column and washed with 50 mL of buffer A containing 0.3 M NaCl. The enzyme

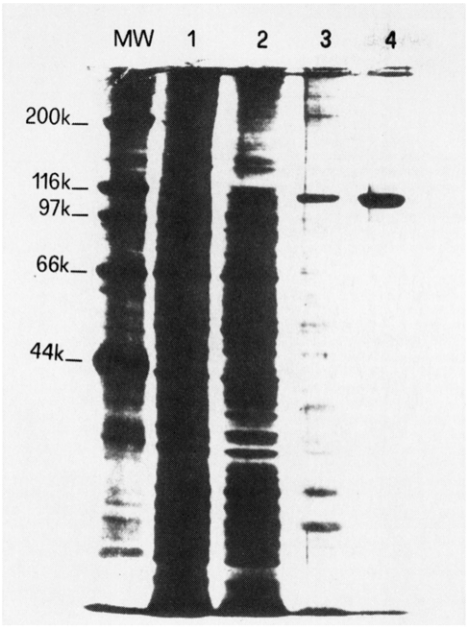

FIGURE 1: SDS gel electrophoresis of the fractions corresponding to the ADPRP purification procedure. Aliquots of the active pools corresponding to the various steps of the purification procedure were analyzed on 10% SDS–PAGE (see Methods). (Lane 1) Crude extract; (lane 2) DNA–agarose fraction; (lane 3) Blue–agarose fraction; (lane 4) final enzyme preparation. The molecular weight markers (Bio-Rad high molecular weight standards) in the outerleft lane are myosin (M_r 200 000), β-galactosidase (M_r = 116 000), phosphorylase B (M_r = 97 000), BSA (M_r = 66 000), and ovalbumin (M_r = 44 000).

Table I: Purification of Poly(ADP-ribose) Polymerase from Bull Testis[a]

fraction	protein (mg)	total units (units)[b]	yield (%)	sp act. (units)/mg	purifn (x-fold)
crude extract	11400	125.4	100	0.011	1
DNA–agarose	542.5	64.1	51.2	0.12	11
Blue–agarose	88.1	29.1	23.2	0.33	30
3-ABA–Sepharose	1.12	15.0	12	13.4	1218

[a] Starting from 200 g of tissue. [b] One unit equals 1 nmol of ADP-ribose incorporated/min in the standard assay conditions (see Methods).

was eluted by using 30 mL of the same buffer containing 2 mM 3-MBA as a competing ligand. Both 3-ABA and 3-MBA are inhibitors of the ADPRP activity (Table IIA). The inhibitor was removed and the enzyme concentrated through a DNA–agarose column as described above. The final enzyme preparation was dialyzed against 80 mM Tris-HCl, pH 8, 10% glycerol (3-ABA–Sepharose fraction) for further characterization studies.

Results and Discussion

Purification of ADPRP. For the purification procedure, see Methods. Figure 1 shows the electrophoretic pattern on 10% SDS–PAGE during the various purification steps. The last chromatographic step yielded a homogeneous, active fraction with an apparent molecular weight of 116 000, corresponding to that described by other authors for the ADPRP from different sources (Burthscher et al., 1986; Kristensen & Holtlund, 1978). The specific activity of the final enzyme preparation was 13.4 nmol min^{-1} mg^{-1} in the standard assay conditions, using histones as the substrate. The complete procedure is summarized in Table I. It has the advantage of being rapid and reproducible, thus suited for the present study, dealing with two relatively unstable enzymes, to keep preparation and storage times to a minimum.

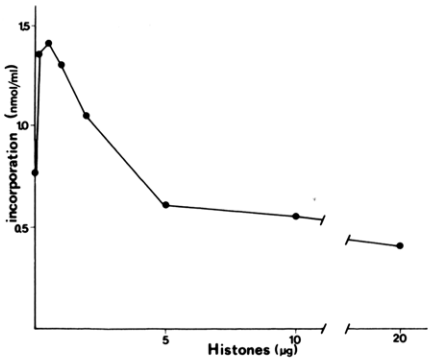

FIGURE 2: Histone dependence of the poly(ADP-ribosylation) reaction. The reaction mixture (0.1 mL) contained 80 mM Tris-HCl, pH 8, 8 mM DTT, 10 μM [^{32}P]NAD (400 000 cpm), 10 mM $MgCl_2$, ADPRP enzyme (0.75 μg), activated DNA (2 μg), and different amounts of histones as shown in the abscissa. The incubation was conducted at 25 °C for 10 min. The reaction was stopped and the TCA-insoluble incorporated radioactivity measured as described under Methods.

Table II: Characterization of the Poly(ADP-ribose) Polymerase Reaction

	reaction mixture	addition	incorporation cpm	incorporation %
A[a]	complete		133 300	100
	complete	+3-ABA (2 mM)	250	0
	complete	+3-MBA (2 mM)	100	0
	complete, −DNA		950	0
	complete, −histones		38 300	29
	complete, $-Mg^{2+}$	+histones	130 000	98
	complete, $-Mg^{2+}$	−histones	37 600	28
B[b]	complete, −histones	+histones	129 600	98
	complete, −DNA	+DNA	128 800	97
	complete, −ADPRP	+ADPRP	129 000	97

[a] Complete reaction mixture was as described under Methods, including 10 μM [^{32}P]NAD (400 000 cpm). Reaction was conducted for 60 min at 25 °C and the enzyme activity measured as the TCA-insoluble incorporated radioactivity. [b] The reaction mixture as above, omitting the indicated component, was preincubated 5 min at 25 °C; the missing component was then added and the reaction conducted for 60 min at 25 °C. Figures represent the average of five independent assays.

Characterization of Purified ADPRP. The activity of the homogeneous enzyme was tested with respect to the histones, DNA, and magnesium ion concentration dependence, to assess the optimal conditions for the modification of exogenous acceptor protein.

Histones and DNA Dependence. At fixed DNA concentration the enzyme exhibited a bell-shaped curve of activity versus histone concentration, 1:1 (w/w) being the optimal ratio (Figure 2). In the absence of DNA the enzyme activity was completely abolished (Table IIA). At optimal DNA/histone ratio the time course of the reaction appeared to be linear up to 15 min, reaching a plateau after 60 min of reaction. In the absence of histones the automodification reaction leveled off after 15 min (Figure 3). Unlike previously reported results (Sastry & Kun, 1988), the activity was not dependent upon a critical order of addition of the macromolecular components of the reaction: DNA, histones, and enzyme. In fact, no variation of the enzyme activity was detected when the assay was performed by preincubating, 5 min at 25 °C, two of the components above in the complete reaction mixture, before adding the last one (Table IIB). Therefore, we could rule out the formation of a dead-end enzyme–histone binary complex in the present case.

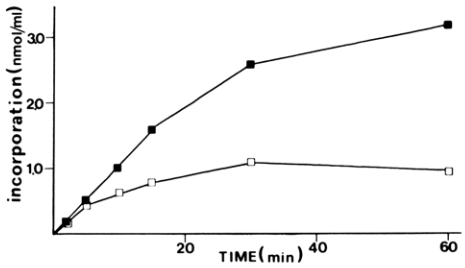

FIGURE 3: Time course of the poly(ADP-ribosylation) reaction. The reaction mixture as in Figure 2, except for ADPRP enzyme, 1.5 μg, and activated DNA, 4 μg, in a final volume of 0.2 mL, was incubated at 25 °C either in the absence (□) or in the presence (■) of 2 μg of histones; 30-μL aliquots were withdrawn at the indicated times, and the TCA-insoluble material was collected on a glass fiber filter and counted as described under Methods.

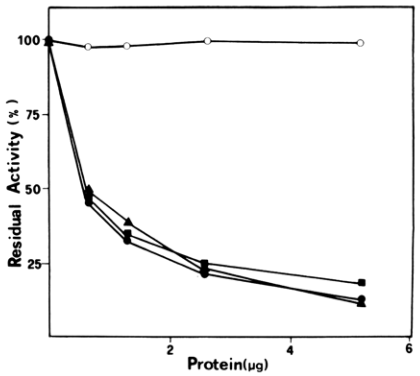

FIGURE 4: Inhibitory effect of NMNAT on the ADPRP activity. Poly(ADP-ribosylation) reaction was performed in the standard assay mixture (see Methods) in the absence of histones and magnesium ion and in the presence of increasing amounts of protein, as indicated in the abscissa. (▲) Native NMNAT; (■) heat-inactivated (100 °C, 10 min) NMNAT; (●) alkali-treated NMNAT (a sample of NMNAT was made 0.1 M KOH in dimethylsulfoxide/water/ethanol (5:4:1) and incubated at 25 °C for 60 min, followed by extensive dialysis against 80 mM Tris-HCl, pH 8, 10% glycerol, prior to the inhibition test); (○) BSA, dissolved in 80 mM Tris-HCl, pH 8, 10% glycerol. In the ordinate is reported the percent residual activity relative to the uninhibited reaction (100%, corresponding to about 1 nmol/min, per milliliter of assay mixture).

Role of Mg^{2+}. Contrasting evidence regarding the modulatory effect of Mg^{2+} on the enzyme activity has been reported (Tanaka et al., 1985; Ushiro et al., 1987; Ueda et al., 1982). In our case the reaction rate appears to be almost completely independent upon the addition of magnesium both in the presence and in the absence of histones (Table IIA).

Poly(ADP-ribosylation) in the Presence of NMNAT. To test NMNAT as a possible poly(ADP-ribose) acceptor, experiments were carried out by incubating NMNAT at various concentrations, as the acceptor, in the presence of the enzyme ADPRP, as detailed in Figure 4. Both enzymes were dialyzed extensively against the same buffer, 80 mM Tris-HCl, pH 8, containing 10% glycerol, previous to the assays. The incubation was carried out in the absence of histones as described (Tanaka et al., 1985) with concentrations of NMNAT ranging from 0.65 to 5.2 μg (0.8–6.4 molar excess with respect to the ADPRP). The reaction was terminated after 20 min and the overall incorporation measured as the acid-precipitable radioactivity. As shown in Figure 4, a pronounced decrease of the incorporation was observed with increasing NMNAT concentrations, evidencing an apparent inhibitory effect of NMNAT over the ADPRP activity. To test whether or not the catalytically active enzyme was required for the inhibition, NMNAT was subjected to heat inactivation prior to the assay. Alternatively, to test whether the carbohydrate bound to the NMNAT protein (Natalini et al., 1986) might be responsible

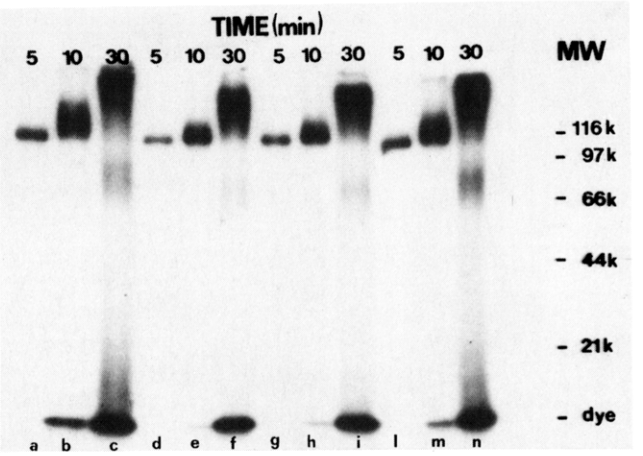

FIGURE 5: Autoradiography after SDS–PAGE of the products of the poly(ADP-ribosylation) reaction in the presence of NMNAT. The reaction was carried out as described under Methods, including 10 μM [^{32}P]NAD (400 000 cpm), histones (2 μg), and various concentrations of NMNAT as specified below; 30-μL aliquots were withdrawn at 5, 10, and 30 min, and the reaction was stopped by the addition of 30 μL of 25% TCA. The acid precipitate was collected by centrifugation and analyzed on 10% SDS–PAGE. Autoradiography was obtained by exposure of the dried gel on Kodak X-Omat film. Lanes a–c correspond to the control, in the absence of NMNAT, at 5, 15, and 30 min of incubation, respectively; the successive lanes correspond to the following decreasing concentrations of NMNAT: 0.375 μM (d–f), 0.250 μM (g–i), and 0.125 μM (l–n). Figures on top refer to incubation times.

for the inhibitory effect, the NMNAT was subjected to mild alkaline treatment (β-elimination) (Downs & Pigman, 1976) and then extensively dialyzed prior to the inhibition assay, as detailed in Figure 4. As shown in the same figure, both the heat-treated and the alkali-treated samples exhibited identical inhibitory effect on the ADPRP activity as the untreated NMNAT, indicating that the inhibiting factor is stable under these conditions. The same pattern was obtained both in the presence and in the absence of 10 mM $MgCl_2$ (not shown).

For the purpose of the autoradiographic identification of the products of the inhibited reaction, an analogous experiment was conducted in the presence of histones, either in the absence or in the presence of NMNAT as detailed in Figure 5. In the absence of NMNAT (Figure 5, lanes a–c), the time course that could be expected for the ADP-ribosylation of ADPRP itself was observed, including a band at M_r = 76 000 corresponding to a proteolytic fragment of ADPRP (Ferro & Olivera, 1982; Holtlund et al., 1983). In the presence of NMNAT (lanes d–n), no additional band in the region of M_r = 50 000 was evidenced, as expected for ADP-ribosylated NMNAT subunits (M_r = 50 000) (Natalini et al., 1986). Such a result indicates that, under the experimental conditions used, NMNAT is not ADP-ribosylated and confirms its inhibitory effect on the ADPRP activity, as evidenced by the band intensities. To investigate the mechanism of the inhibition, several possibilities were examined: (1) formation of a stable, inactive complex between NMNAT and ADPRP; (2) formation of a complex between NMNAT and DNA, removing DNA from the reaction; (3) NAD consumption through unknown side reactions.

(*1*) To detect the formation of a stable ADPRP–NMNAT complex, gel filtration chromatography on Superose 12 FPLC column was used. Various mixtures, containing different ADPRP/NMNAT molar ratios either incubated in a complete reaction mixture or not, were run separately on the same column. Both the elution volume and the recovery of the NMNAT enzymatic activity were found in all cases indistinguishable from those of the control, represented by

NMNAT alone. In addition, no shift of the elution profile such to indicate the formation of a high molecular weight complex was evidenced. These results, thus, appear to rule out a dead-end stable enzyme–enzyme complex as a basis for the inhibition.

(*2*) An NMNAT–DNA interaction was either tested by binding experiment or tested indirectly by the ability of DNA to remove the inhibition. Direct binding measurement was performed by membrane binding assay according to the reported procedures (Maniatis et al., 1982; Freifelder, 1982; Ohgushi et al., 1980). By use of radioactive DNA and NMNAT immobilized onto nitrocellulose membrane, no radioactive incorporation on the membrane was observed. Furthermore, to test for the possible occurrence of competition between the two enzymes for activated DNA, resulting in an impaired availability of DNA for the enzyme activation, the inhibition experiments were performed in the presence of increasing concentrations of activated DNA, either in the absence of histones or in the presence of histones and DNA at a constant ratio (1:1). The results showed that an increase of the DNA concentration up to 10-fold failed to restore the original ADPRP activity.

Furthermore, NMNAT could affect the reaction by preventing the correct arrangement of the catalytic complex (Sastry & Kun, 1988): since the occurrence of a dead-end ADPRP–histone complex has been ruled out in the present case, this hypothesis was tested by preincubating for 5 min at 25 °C in the standard assay mixture three of the macromolecular components (DNA, ADPRP, and either the histones or the NMNAT) before the fourth component was added, either NMNAT or histones, respectively. The results showed the same degree of inhibition, irrespective of the order of addition (not shown).

(*3*) The third possibility, i.e., depletion of NAD through side reactions, was investigated either directly, by analyzing the reaction mixture following the assay, or indirectly, by increasing the NAD concentration in the reaction mixture. Under conditions yielding over 80% inhibition, unexpected byproducts were not detected following electrophoresis on cellulose thin layer (Atkinson et al., 1961), and the original amount of radioactivity was found to correspond to the unreacted NAD and protein-bound radioactivity.

To test whether an increase of the NAD concentration could prevent the inhibition, the NAD concentration was increased, up to a 600-fold molar excess over NMNAT. As a result the inhibition was not significantly affected over the range tested.

The above results demonstrate that the observed inhibition is apparently not due to the depletion of an essential reaction component.

The procedure described in the present work allows the rapid obtaining of homogeneous and active ADPRP, suitable for the ADP-ribosylation of exogenous protein acceptors. From the evidence presented, it appears that pure yeast NMNAT exerts an inhibitory effect on the reaction catalyzed by ADPRP, instead of the stimulatory effect which might be expected if, according to our previous findings (Natalini et al., 1986; Ruggieri et al., 1988), the NMNAT was to be poly(ADP-ribosylated). However, although the modification of specific enzyme acceptors by means of heterologous polymerase from completely unrelated organisms has been reported (Taniguchi et al., 1985; Tanaka et al., 1985), the requirement for the homologous polymerase might be stringent for the yeast enzyme.

On the other hand, albeit positive evidence for the presence of the ADPRP activity in yeast has been shown (Miwa et al., 1983; Mura, 1987; Ruggieri et al., 1988), the observed inhibition may be regarded as somehow disjoined from the yeast system, possibly representing a novel feature of the poly(ADP-ribosylation) reaction, and a particular case of control of sequential metabolic enzymes (Srere, 1987; Ovadi, 1988). In this regard the present findings may appear suggestive also in the light of the observations that ADPRP and NMNAT seem to exist in a 1:1 relationship within the chromatin (Uhr & Smulson, 1982) and that most of the ADPRP may be in a catalytically inactive state in situ (Yamanaka et al., 1988). Studies are in progress in our laboratory to elucidate the significance of the observed inhibition and the possibility that NMNAT is ADP-ribosylated under appropriate experimental conditions, including the use of a homologous enzyme system.

Registry No. NMNAT, 9032-70-6; ADPRP, 9055-67-8; Mg, 7439-95-4.

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Vasotocin Genes of the Teleost Fish *Catostomus commersoni*: Gene Structure, Exon–Intron Boundary, and Hormone Precursor Organization[†,‡]

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ABSTRACT: cDNA clones encoding two members of the vasotocin hormone precursor gene family have been isolated from the white sucker *Catostomus commersoni*. The hormone is encoded by at least two distinct genes, both of which are expressed, as indicated by Northern blot analysis. Genomic DNA amplified by the polymerase chain reaction has been used to define exon–intron boundaries. Both vasotocin genes contain introns in positions corresponding to those found in the gene of their mammalian counterpart vasopressin. The predicted vasotocin precursors show a surprising degree of sequence divergence, amounting to 45% at the amino acid level, of which only approximately half can be accounted for by conservative amino acid changes. The precursors include a hormone moiety followed by a putative neurophysin sequence that is longer at the C-terminus by a tract of some 30 amino acids by comparison to their mammalian counterpart. Each of these sequences contains a leucine-rich core segment resembling that found in copeptin, a glycopeptide moiety present in mammalian vasopressin precursors.

Neuropeptides were probably among the earliest molecules to play a signaling role in the evolving nervous system. Initially it had been assumed that regulatory peptides, such as vasopressin and oxytocin, would be found exclusively within vertebrates [reviewed in Krieger (1983)], where they can function as endocrine hormones, neurotransmitters, and paracrine factors [reviewed in Hoffman (1987) and Ishikawa et al. (1987)]. A wealth of studies have shown, however, that vasopressin and oxytocin are the predominant mammalian homologues of a larger, structurally conserved, family of neuropeptide hormones (Table I), whose members are distributed throughout the animal kingdom, including groups as diverse as molluscs and insects [reviewed in Acher and Chauvet (1988)]. These peptides all consist of nine amino acids with cysteine residues in positions one and six forming a disulfide bridge. While Asn[5], Pro[7], and the C-terminal glycine amide are also completely conserved, amino acid variations exist at positions 2, 3, and 4, giving rise to the different family members shown in Table I and, most significantly, at position 8 where the change from Leu or a related lipophilic amino acid to Arg or Lys accounts for a vasopressin-type as opposed to an oxytocin-type function (Hruby & Smith, 1987). In all

Table I: Vasopressin–Oxytocin Neuropeptide Hormone Family[a]

OXYTOCIN FAMILY		
CYS TYR ILE GLN ASN CYS PRO LEU GLY·NH2	*OXYTOCIN*	*mammal*
CYS TYR ILE GLN ASN CYS PRO ILE GLY·NH2	*MESOTOCIN*	*non-mammalian tetrapod*
CYS TYR ILE SER ASN CYS PRO ILE GLY·NH2	*ISOTOCIN*	*bony fish*
CYS TYR ILE SER ASN CYS PRO GLN GLY·NH2	*GLUMITOCIN*	*cartilagenous fish*
CYS TYR ILE GLN ASN CYS PRO VAL GLY·NH2	*VALITOCIN*	
CYS TYR ILE ASN ASN CYS PRO LEU GLY·NH2	*ASPARGTOCIN*	
VASOPRESSIN FAMILY		
CYS TYR PHE GLN ASN CYS PRO ARG GLY·NH2	*ARG·VASOPRESSIN*	*mammal*
CYS TYR PHE GLN ASN CYS PRO LYS GLY·NH2	*LYS·VASOPRESSIN*	
CYS PHE PHE GLN ASN CYS PRO ARG GLY·NH2	*PHENYPRESSIN*	
CYS TYR ILE GLN ASN CYS PRO ARG GLY·NH2	*VASOTOCIN*	*non-mammalian vertebrate*
CYS PHE ILE ARG ASN CYS PRO LYS GLY·NH2	*LYS·CONOPRESSIN*	*mollusc (Conus)*
CYS ILE ILE ARG ASN CYS PRO ARG GLY·NH2	*ARG·CONOPRESSIN*	*mollusc (Conus)*
CYS LEU ILE THR ASN CYS PRO ARG GLY·NH2	*DIURETIC HORMONE*	*insect (Locust)*

[a] The cysteine residues are linked by a disulfide bridge. Mammals: in general, oxytocin and arginine vasopressin are found in mammals except in pig which has lysine vasopressin and the Methatherian marsupials which have mesotocin, lysine vasopressin, and phenypressin either in addition or as alternatives to their normal mammalian counterparts. Nonmammalian tetrapods (birds, reptiles, and amphibians): mesotocin and vasotocin. Bony fish (e.g., sucker): vasotocin and isotocin. Cartilaginous fish: vasotocin and glumitocin (rays), vasotocin, valitocin, and aspargtocin (sharks). Mollusc: in fish-hunting snails (*Conus*), two peptides have been identified, namely, lysine conopressin and arginine conopressin. Locust: *Locusta migratoria* contains an arginine vasopressin like diuretic hormone. Compiled from Proux et al. (1987), Acher and Chauvet (1988), and Gray et al. (1988).

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