ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 3, pp. 289-295. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 3, pp. 356-363.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-322, January 7, 2008.

Isolation and Physicochemical Properties of Tankyrase of Human Embryonic Kidney Cells of Line 293

N. N. Sidorova[1], A. O. Fadeev[2], and A. N. Kuimov[1]*

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: kuimov@genebee.msu.su*
[2]*Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, 119992 Moscow, Russia*

Received September 24, 2007
Revision received October 9, 2007

Abstract—We have isolated and purified endogenous cytosolic tankyrase from human embryonic kidney cells of line 293. Our data confirm a model of De Rycker and Price who consider that tankyrase is a master scaffolding protein capable of regulating assembly of large protein complexes. We have also studied kinetic characteristics of tankyrase in the complex, pH dependence of the enzyme activity, and its physicochemical properties.

DOI: 10.1134/S0006297908030085

Key words: tankyrase, poly(ADP-ribose), PARP, ADP-ribosylation, kidney

In the human genome the poly(ADP-ribose) polymerase (PARP) superfamily consists of 17 proteins, which share a homologous PARP catalytic domain and presumably enzyme activity (EC 2.4.2.30) of autocatalytic poly(ADP-ribosylation) as well as the same modification of a number of specific protein substrates [1]. All studied PARPs use also NAD^+ as a second substrate to build poly(ADP-ribose) chains. The first known member of the superfamily is PARP-1, a nuclear protein, which is activated by DNA damage to participate in DNA repair. Recently two more members named tankyrases 1 and 2 were also studied. The first of these was initially found on telomeres, where human tankyrase 1 controls its covering protein complex shelterin [2, 3], but later both tankyrases were also localized in cytoplasm as interacting partners of a number of other proteins [4-8]. On the other hand, in mice tankyrase had no effect on shelterin, and the murine enzyme functions in other subcellular compartments but not in the nucleus [3, 9].

In our studies of tissue distribution of human tankyrase 2, the protein was found in a few normal tissues, namely epithelium of renal tubules and small intestine, although it was also abnormally expressed in some breast tumors, while most tumors as well as normal breast tissues had no tankyrase 2 [10, 11]. Tissue specific expression was later reported for murine tankyrase [9]. Human intestinal epithelium contained tankyrase 2 in Golgi, as previously described for murine tankyrase [5]. Renal epithelial cells had the protein in the whole cytoplasm. We also discovered that human embryonic kidney cells of line 293 had much more tankyrase than any other studied cell lines. Almost all the enzyme was located in the cytoplasm, and about 50% of it was soluble, while the rest was associated with membrane fractions [10]. Same subcellular distribution was documented for tankyrase in chicken tumor cells [12, 13].

The study of chicken tankyrase also revealed that the cytosolic enzyme had a high molecular mass as if it was associated in a polymer or complex. Autocatalytic PARP activity of tankyrase resulted in disassembly of these polymers [13]. This fact may be a clue for understanding of the physiologic function of cytosolic tankyrase and its role in tumor development.

Since tankyrase is not uniformly distributed in various organs and tissues, we suggested that this signaling molecule might have different functions in each tissue, and therefore different protein substrates. In this study, we have worked with cytosolic tankyrase of kidney cells, which are the richest known source of the enzyme, and found that it is also assembled in large partially soluble aggregates. We have isolated these aggregates, which have complex composition, and plan to study this composition in further investigation in order to determine the function of tankyrase in kidney.

Abbreviations: PARP) poly(ADP-ribose) polymerase.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparation of human cells. Human embryonic kidney cell line 293 was grown in DMEM [14] containing 10% (v/v) fetal bovine serum. Twenty 100-mm plates of confluent cells were used per experiment. The cells were washed on plates once with Hanks' balanced salt solution [14], once with ice-cold TBS (Tris-HCl, 20 mM; NaCl, 150 mM; pH 7.6), and were harvested in ice-cold TBS.

Isolation of the soluble tankyrase 2. All the following procedures were conducted at 4°C. The cell suspension was centrifuged at 400*g* for 10 min at 4°C, and the supernatant was discarded. The pellet was resuspended in an equal volume of buffer F (Tris-HCl, 10 mM; sucrose, 0.25 M; EDTA, 0.5 mM; pH 7.6) [5, 6] with protease inhibitor cocktail (Amresco, USA) and centrifuged at 5000*g* for 5 min at 4°C. The supernatant was separated, while the pellet was homogenized in a loose-fitting Teflon–Teflon homogenizer, resuspended in the same supernatant, and centrifuged again at 5000*g* for 5 min at 4°C. The first extract was separated, while the pellet was homogenized again in an equal volume of fresh ice-cold buffer F with protease inhibitor cocktail and centrifuged as described above. The pellet was discarded, while the second extract was combined with the first and centrifuged at 20,000*g* for 5 min at 4°C. The pellet was discarded, and the supernatant was filtered via a cellulose filter to remove lipid particles. An aliquot was used to assay protein [15, 16] and PARP activity (see below), and the crude extract was loaded onto a column (25 × 25 mm) with DEAE-cellulose (Amersham Pharmacia Biotech, Sweden) equilibrated with TBS, washed with 20 ml of TBS, and the protein was recovered with 20 mM Tris-HCl, pH 7.6, which contained 0.4 M NaCl. Elution of nucleoprotein was detected using optical density at 260 nm. An aliquot was taken again as well as during further steps to assay protein [15, 16] and PARP activity. For further purification, the rest of preparation was used in the following chromatography procedures.

Chromatography of Sepharose 2B. The fractions with peak PARP activity collected during the previous step were combined in a total volume of no more than 5 ml, loaded on a column (350 × 25 mm) with Sepharose 2B (Amersham Pharmacia Biotech) equilibrated with TDN (Tris-HCl, 20 mM; DTT, 0.1 mM; Nonidet P-40, 0.02%; pH 7.6), with or without NaCl and eluted with the same buffer. The column was calibrated with dextran blue, thyroglobulin (Fluka, USA), and IgG (ICN Biomedicals, Inc., USA). Elution of the enzyme was also detected using optical density at 260 nm and PARP activity assay.

Chromatography on Mono Q column. Fractions containing major 130-kD PARP substrate were collected and loaded onto a Mono Q HR 10/10 column (Amersham Pharmacia Biotech) equilibrated with buffer IS (imidazole, 250 mM; NaCl, 75 mM; $MgCl_2$, 2.5 mM; EDTA, 0.5 mM; pH 7.6). The column was washed with buffer IS until the protein disappeared in the eluate. Then the column was washed with 20 ml of buffer TED (Tris-HCl, 20 mM; EDTA, 0.5 mM; DTT, 0.1 mM; pH 7.6), and the 130-kD PARP substrate was recovered in a 0.15-1.0 M NaCl gradient in buffer TED. The enzyme fractions were mixed with equal volume of saturated solution of ammonium sulfate, pH 7.6, with 1 mM EDTA, and protease inhibitor cocktail. The preparation of partially purified tankyrase could be stored at 4°C as a suspension for up to one month.

Rechromatography on Sepharose 2B. For this step of purification, the suspension in ammonium sulfate was mixed with one more volume of saturated solution of ammonium sulfate, pH 7.6, and centrifuged at 20,000*g* for 5 min at 4°C. The supernatant was discarded, and the pellet was dissolved in 0.5 ml of TDN with 150 mM NaCl and 10 mM EDTA, centrifuged once again to remove insoluble material, loaded on a column (250 × 8 mm) with Sepharose 2B (Amersham Pharmacia Biotech) equilibrated with TDN buffer with 150 mM NaCl and 1 mM EDTA, and eluted with the same buffer. Elution of the enzyme was detected using optical density at 280 nm and PARP activity assay.

Chromatography on Superose 12. The disassembly of tankyrase complex was detected using chromatography on Superose 12 10/300 GL (Amersham Pharmacia Biotech) equilibrated with TDN buffer with 150 mM NaCl and 1 mM EDTA. The enzyme preparation from the previous step was concentrated using a Centricon Ultracel YM-30 filter device (Millipore, USA) and used for PARP assay for up to 2 h at 25°C. The volume of the reaction mixture was 0.5 ml. An inhibitor niacinamide (Sigma, USA), 10 mM, was added into a parallel sample, which was used as a negative control. After the incubation, the reaction mixture was loaded on a Superose 12 column and eluted with TDN buffer with 150 mM NaCl and 1 mM EDTA. The column was calibrated with IgG and ovalbumin (ICN Biomedicals, Inc.). Elution of the protein was detected using optical density at 280 nm.

PARP activity assay. To assay the tankyrase enzyme activity, a method of autocatalytic poly(ADP-ribosylation) was used [2, 5, 10]. An enzyme preparation was incubated at 25°C in reaction buffer containing 125 mM imidazole, 40 mM NaCl, 4 mM $MgCl_2$, 0.2 mM EDTA, 20 μM [^{32}P]NAD^+ (10 μCi/ml), protease inhibitor cocktail; pH 7.6 or as indicated in the text. Preparation of [^{32}P]NAD^+ had been synthesized and kindly provided by Yu. S. Skoblov (V. A. Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). For chromatography experiments, non-radioactive NAD^+ (ICN Biomedicals, Inc.) was also used instead of the radioactive NAD^+. An inhibitor niacinamide, 10 mM, was added into a parallel sample, which was used as a negative control. When incubation was completed, the products were analyzed using chromatography or electrophoresis. For

the latter method, the reaction was stopped with ice-cold acetone mixed in a ratio 1 : 1 (v/v). Protein was precipitated during incubation for 30 min at −18°C and following centrifugation at 18,000*g* for 5 min at 4°C. Supernatant was discarded, and the pellet was resuspended in a sample buffer (Tris-HCl, 125 mM, pH 6.8; SDS, 1 mg/ml; glycerol, 10% (v/v); dithiothreitol, 15 mg/ml; bromophenol blue, 0.01 mg/ml), boiled for 3 min, and loaded onto 7% polyacrylamide gel [17].

Protein electrophoresis. Electrophoresis under denaturing conditions (SDS-PAGE) was performed at room temperature as previously described [10]. Protein molecular weight markers (Amresco) were loaded in one of the lanes. When SDS-PAGE was completed, a gel was used for silver staining [18] or transfer of the proteins to nitrocellulose (Osmonics Inc., USA) [10] to visualize tankyrase 2 using antibodies or PARP assay. The nitrocellulose membrane was then stained with Ponceau S (Sigma) to check the quality of transfer and used for autoradiography or Western blots.

Western blots. The nitrocellulose membrane was blocked with SuperBlock Dry Blend Blocking Buffer in TBS (Pierce, USA) with 3% (w/v) bovine serum albumin, incubated with the affinity-purified polyclonal antibody [11] diluted in TBST (TBS with 0.1% Triton X-100), vigorously washed with TBST, incubated with goat anti-rabbit secondary antibodies (Biosource, USA) conjugated with horseradish peroxidase, washed again, incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce), and exposed to X-ray film for visualization of the signal.

Autoradiography. The nitrocellulose membrane was dried and exposed to a BAS-MS imaging plate (Fujifilm, Japan). The signal was detected and quantified using a FLA-3000 imaging analyzer (Fujifilm).

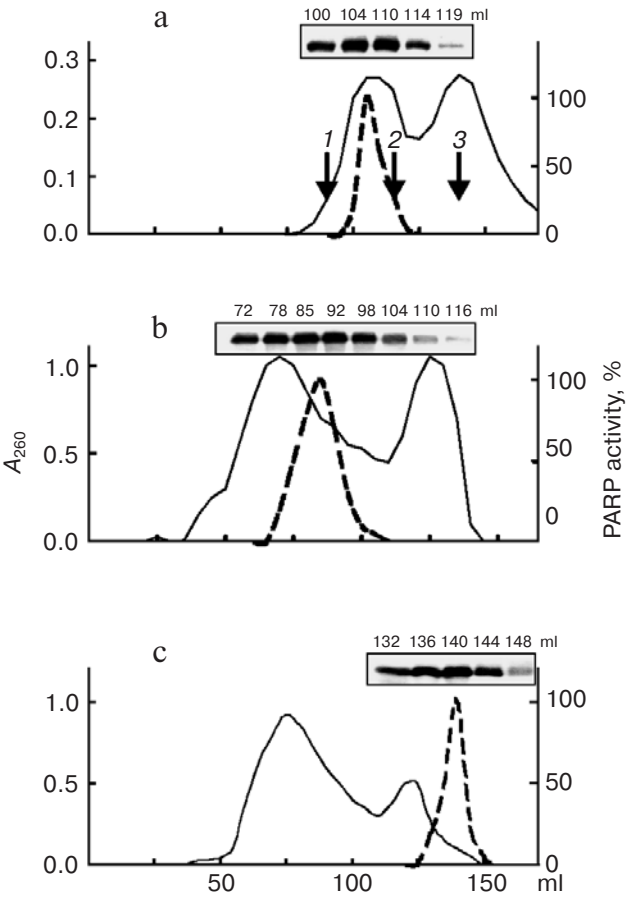

Fig. 1. Elution of PARP activity from Sepharose 2B. a) Elution under physiological conditions (TDN buffer with 150 mM NaCl). Arrows *1-3* indicate positions of molecular weight markers—dextran blue (2000 kD), thyroglobulin (670 kD), and IgG (150 kD), respectively. b) Elution at low ionic strength (TDN buffer without NaCl). c) Elution at low ionic strength after reaction of poly(ADP-ribosylation). Solid lines, detection of optical density at 260 nm; dashed lines, PARP activity assay. Electrophoresis of major 130-kD PARP substrate (tankyrase 2) in selected fractions is shown above.

RESULTS

In our previous study, we found that the major PARP enzyme in cytosol of line 293 embryonic kidney cells is tankyrase 2 [10]. To determine its molecular mass *in vivo*, we used gel filtration of lysate of 293 cells on Sepharose 2B. Under physiologic conditions (buffer TDN with 150 mM NaCl), we observed elution of PARP activity in the range 500-2000 kD (Fig. 1a) as previously observed with chicken tankyrase [13]. On SDS-PAGE gel the mass of the major poly(ADP-ribosylated) product was about 130 kD, which is the size of tankyrase 2 monomer, while its mass during gel filtration looks like that of an oligomer or a complex with some other molecules. At low ionic strength (buffer TDN without NaCl), we saw even larger entities (Fig. 1b), which means that the observed 130-kD PARP substrate can aggregate, and this process depends on electrostatic charge of the molecules.

After incubation with NAD^+ under conditions of PARP activity assay, the molecular mass of the major PARP substrate identified by gel filtration became much less (Fig. 1c). The mass did not depend on ionic strength, so the poly(ADP-ribosylated) 130-kD product did not seem to aggregate under any conditions.

We used the aggregation of the major PARP substrate at various ionic strengths for its further purification in a procedure that allowed collection of fractions of high molecular weight during first gel filtration and fractions of medium molecular weight during second one. This way the impurities of different molecular mass can be effectively removed.

The table contains data on the purification of the 130-kD PARP substrate during this procedure. At physiological pH value, the enzyme of interest can be bound by

Purification of tankyrase from human embryonic kidney cells of line 293 (a representative experiment)

Step	Total protein, mg	Specific activity, nmol/min per mg protein	Recovery, %*
Lysate	24	0.0006	100
DEAE-cellulose	7.35	0.0016	86
Gel filtration on Sepharose 2B	1.9	0.0033	45
Mono Q	0.37	0.0092	24
Gel filtration on Sepharose 2B	0.033	0.0455	10.7

* Recovery was calculated based on PARP enzyme activity.

an anion-exchange resin. First, we used DEAE-cellulose to clarify the crude extract. After the first gel filtration on Sepharose 2B at low ionic strength, the fractions containing PARP activity were loaded on the Mono Q column to concentrate and further purify the enzyme preparation. Then the protein was loaded onto the second Sepharose 2B column and eluted under physiological conditions (Fig. 2). The 130-kD PARP substrate was recovered from the Mono Q column in a gradient of NaCl at 0.6-0.8 M salt concentration (Fig. 2a). During rechromatography on Sepharose 2B it was well separated from a main protein peak, which contained the rest of the impurities of high molecular mass (Fig. 2b).

At this step the purified protein changed its molecular mass after poly(ADP-ribosylation). In Fig. 3a the gel filtration on Superose 12 indicates that incubation of the protein with NAD^+ resulted in disassembly of ~1000-kD aggregates isolated during rechromatography on Sepharose 2B. Initially the protein was eluted from Superose 12 in the void volume, but after incubation with NAD^+ some smaller protein molecules could also be eluted, which suggests the disintegration of the initial protein aggregate. First, the molecular mass of product was higher than 150 kD, but further poly(ADP-ribosylation) resulted in complete disassembly of intermediate product to polypeptides of lesser size, the mass of which was anyway no less than 100 kD. The disassembly was completely prevented with niacinamide, an inhibitor of poly(ADP-ribosylation).

The results of purification were also analyzed on SDS-PAGE gel and Western blot (Fig. 3b). Human tankyrase 2 was detected by antibody at all steps of the isolation procedure, which means that the isolated protein contains tankyrase 2. On the other hand, the protein consists of several polypeptides seen at 100-200 kD, and only one of them can be visualized using anti-tankyrase antibody, which suggests that the isolated aggregate may be a complex of several proteins along with tankyrase.

In a study of catalytic activity of the purified preparation, we found that its specific activity was higher than reported earlier for tankyrase 1. Taking into account an average molecular mass of tankyrase complex as 1000 kD, the catalytic constant in our assay was $(8 \pm 5)\cdot10^{-4}$ sec^{-1}, while the same value for tankyrase 1 was $(7 \pm 1)\cdot10^{-6}$ sec^{-1} [19]. The K_m value for NAD^+ was also different, 5 ± 3 μM for our preparation or 1.5 ± 0.8 mM for tankyrase 1 [20]. The dependence of velocity of autocatalytic poly(ADP-ribosylation) on NAD^+ concentration in double reciprocal coordinates is shown on Fig. 3c.

We also studied the dependence of tankyrase activity on pH (Fig. 4). The enzyme was active in a range of pH 6.5-8.5 with a broad maximum at pH 7.2-7.8.

DISCUSSION

Since the discovery of tankyrase ten years ago [2], nobody published a procedure for purification of endogenous enzyme. First, tankyrase was found in a nucleoprotein complex of telosome and later as a membrane-bound protein [5], which made very difficult its biochemical purification. Secondly, the enzyme could be expressed using a baculovirus system [2] or obtained by immunoprecipitation [5]. Both ways had their disadvantages. The baculovirus-derived enzyme had very low catalytic activity [2, 19, 20], probably because of lack of endogenous effectors, while immunoprecipitation could not provide tankyrase free from antibodies, since its recovery from the precipitate was not successful.

Our two recent findings allowed an attempt for purification of endogenous tankyrase. We found that

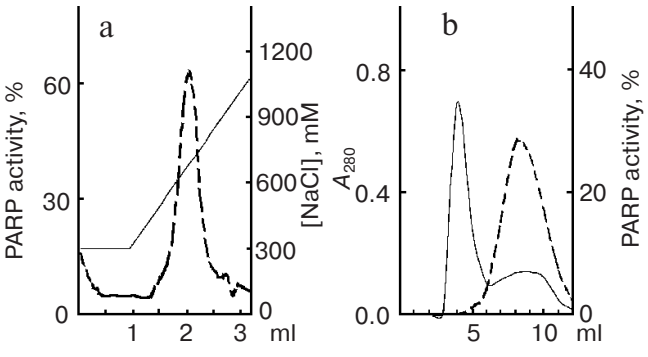

Fig. 2. Elution of PARP activity from Mono Q (a) and rechromatography on Sepharose 2B (b). a) Enzyme preparation eluted from Sepharose 2B (Fig. 1b) was loaded on a Mono Q column and recovered in a gradient of NaCl (solid line). b) Preparation eluted from Mono Q was loaded on Sepharose 2B and eluted at high ionic strength (TDN buffer with 150 mM NaCl). Solid line, detection of optical density at 280 nm; dashed line, PARP activity.

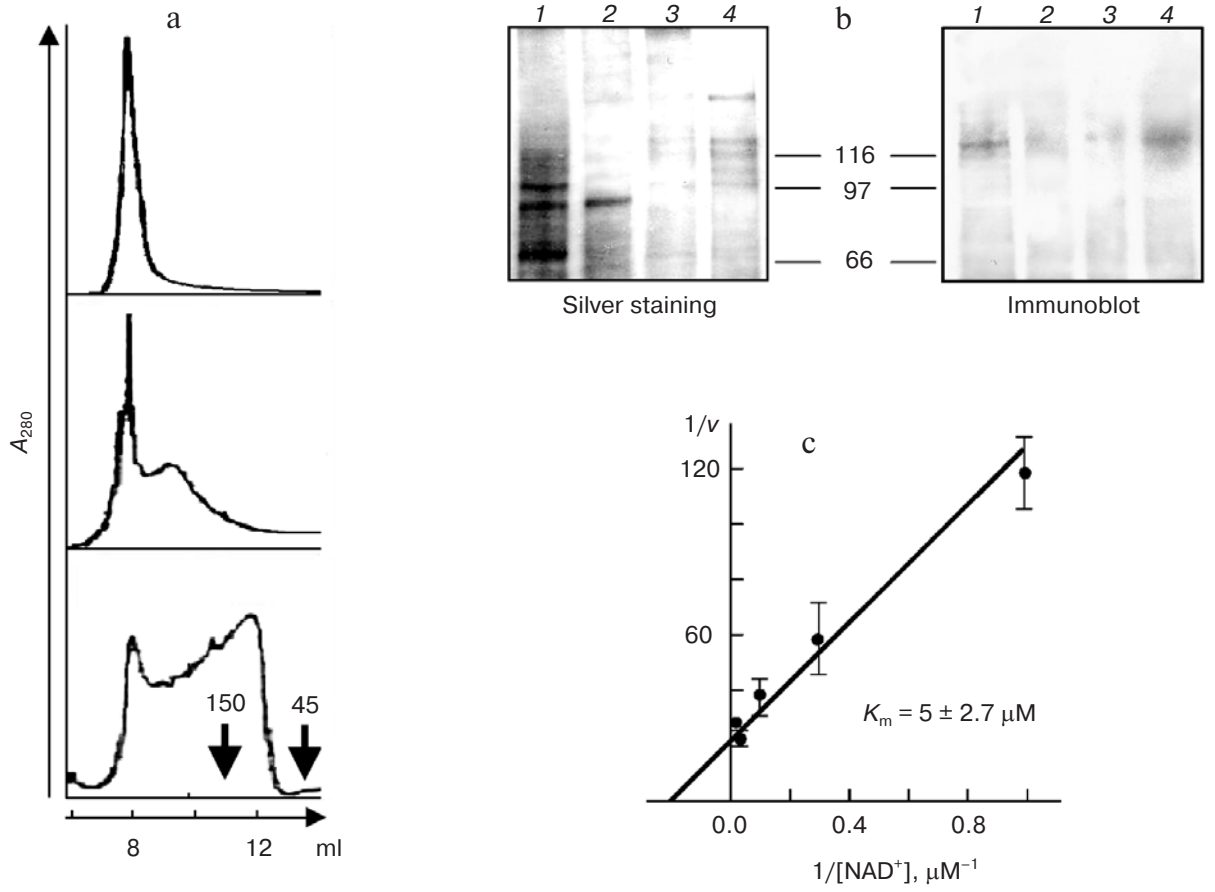

Fig. 3. Characterization of purified tankyrase preparation. a) Elution from Superose 12 before (upper panel) or after poly(ADP-ribosylation) in 30 min (middle panel) or 2 h (lower panel). Detection of optical density at 280 nm. Arrows indicate positions of molecular weight markers: IgG (150 kD) and ovalbumin (45 kD). b) SDS-PAGE (left panel) and Western blot (right panel) of the protein preparation at different steps of purification: *1*) crude cytosolic extract; *2*) eluate from DEAE cellulose; *3*) eluate from the first column with Sepharose 2B; *4*) eluate from the second column with Sepharose 2B. Figures between panels designate positions of markers of molecular mass. c) Kinetic characterization of the purified enzyme preparation by dependence on [NAD^+] of tankyrase PARP activity in double reciprocal coordinates. Three independent experiments were carried out at each point.

human kidney cells of line 293 were a rich source of this enzyme, and a significant part of the enzyme was soluble [10]. This was a rare case, since most other cell types have very low, if any, amount of tankyrase, and this protein is mostly bound in the nucleus, Golgi, or membrane vesicles [2, 4-6]. Soluble tankyrase was also found in chicken hepatocellular carcinoma cell line [13], where its solubility was dependent on concentration and medium.

To avoid a problem of low recovery during immunoprecipitation, in our study we used traditional methods of preparative enzymology. The purification resulted in accumulation of a large ~1000 kD protein complex, which could be dissociated after tankyrase autocatalytic poly(ADP-ribosylation). The same size of soluble tankyrase complex and its dependence on tankyrase activity were reported for chicken tankyrase, both baculovirus-derived and endogenous in crude extract [13]. The complex seems to be stable at both low and high (up to 0.8 M NaCl) ionic strength, which is crucial for electrostatic and hydrophobic interactions. Nevertheless, the subunits of the complex have no covalent bonds between them, since they can be eluted separately after autocatalytic poly(ADP-ribosylation) of tankyrase.

Relatively high enzyme activity and affinity of the enzyme for NAD^+ indicate a good quality of our enzyme preparation. The NAD^+ binding of previously studied overexpressed tankyrase 1 was extremely weak; its K_m was 1.5 ± 0.8 mM [20]. The respective K_m value of our preparation is 5 ± 3 μM, which is only 10 times lower than the K_m value for PARP-1, 50 μM [21]. That means that at least tankyrase 2 binds NAD^+ quite tightly. The question is how can tankyrase complex exist *in vivo*, if tankyrase can bind the substrate, and its poly(ADP-ribosylation) disintegrates the complex? One possible explanation is that the velocity of the process is too low, and poly(ADP-ribosylation) cannot be performed because of rapid hydrolysis of poly(ADP-ribose) in cells.

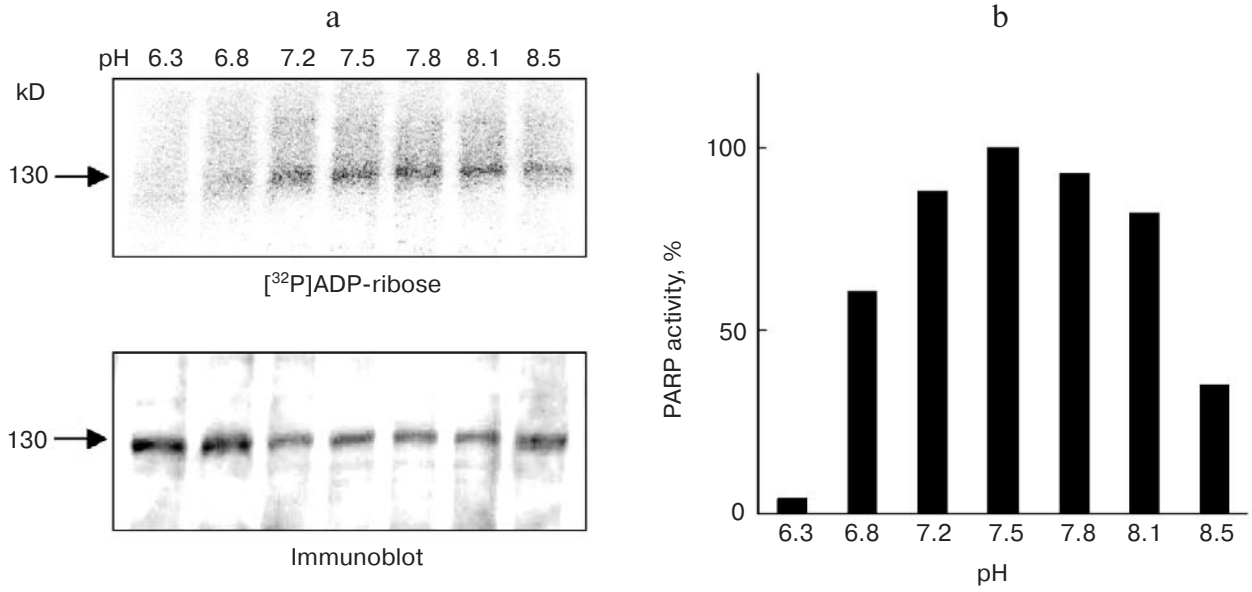

Fig. 4. Dependence on pH of tankyrase PARP activity. a) PARP assay (upper panel) and Western blot (lower panel) of preparations poly(ADP-ribosylated) at various pH values shown above. Arrows indicate positions of 130-kD tankyrase 2 signal. b) Quantitation of tankyrase PARP activity.

Indeed, the k_{cat} value of human tankyrase in purified complex was $(8 \pm 5)\cdot 10^{-4}$ sec^{-1}, which is about 100 times higher than reported for baculovirus-derived tankyrase 1 [19] but still very low in comparison with other enzymes like PARP-1. It is close to the k_{cat} value for non-activated PARP-1, which is 10^{-3} sec^{-1}, and ~500-fold lower than that of PARP-1 activated by DNA breaks [21]. We should also emphasize that our estimation of k_{cat} value depends on the molecular mass of tankyrase complex, which was in average 1000 kD, and during the dissociation of the complex we could detect some intermediates of lower mass (Fig. 3a). The k_{cat} value may be somewhat different for these intermediates, although not dramatically.

We observed several polypeptide chains along with tankyrase on an SDS-PAGE gel (Fig. 3b), which seem to be other subunits of the complex. In summary their mass may be 400-600 kD, which is close to the mass of the intermediate or minimal mass of the endogenous tankyrase complex [13]. We suggest that this ~500 kD unit contains one monomer of tankyrase, and the very soluble tankyrase complex can consists of 2-4 such units. Our model is similar to the model by De Rycker and Price [13], who consider that tankyrase is a master scaffolding protein capable of regulating assembly of large protein complexes.

Our model should still be tested by further experiments, since we do not know what are the other polypeptides in the enzyme preparation, what is their biological function, or even if some of them could be just impurities. In the future, we plan to study other subunits of the complex and their interaction with tankyrase to solve these problems.

The authors highly appreciate help by Yu. S. Skoblov (V. A. Engelhardt Institute of Molecular Biology, Russian Academy of Sciences) for [^{32}P]NAD$^+$ synthesis, Y. E. Dunaevsky (A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University) for assistance in experiments with FPLC equipment, and Carolyn Price (University of Cincinnati) for many valuable discussions.

This study was supported by the Russian Foundation for Basic Research (grant No. 06-04-49388).

REFERENCES

1. Otto, H., Reche, P. A., Bazan, F., Dittmar, K., Haag, F., and Koch-Nolte, F. (2005) *BMC Genomics*, **6**, 139.
2. Smith, S., Giriat, I., Schmitt, A., and de Lange, T. (1998) *Science*, **282**, 1484-1487.
3. Donigian, J. R., and de Lange, T. (2007) *J. Biol. Chem.*, **282**, 22662-22667.
4. Smith, S., and de Lange, T. (1999) *J. Cell Sci.*, **112**, 3649-3656.
5. Chi, N.-W., and Lodish, H. F. (2000) *J. Biol. Chem.*, **275**, 38437-38444.
6. Lyons, R. J., Deane, R., Lynch, D. K., Ye, Z. S., Sanderson, G. M., Eyre, H. J., Sutherland, G. R., and Daly, R. J. (2001) *J. Biol. Chem.*, **276**, 17172-17180.
7. Seimiya, H., and Smith, S. (2002) *J. Biol. Chem.*, **277**, 14116-14126.

8. Sbodio, J. I., and Chi, N. W. (2002) *J. Biol. Chem.*, **277**, 31887-31892.
9. Muramatsu, Y., Ohishi, T., Sakamoto, M., Tsuruo, T., and Seimiya, H. (2007) *Cancer Sci.*, **98**, 850-857.
10. Kuimov, A. N., and Terekhov, S. M. (2003) *Biochemistry (Moscow)*, **68**, 260-268.
11. Sidorova, N., Zavalishina, L., Kurchashova, S., Korsakova, N., Nazhimov, V., Frank, G., and Kuimov, A. (2006) *Cell Tissue Res.*, **323**, 137-145.
12. De Rycker, M., Venkatesan, R. N., Wei, C., and Price, C. M. (2003) *Biochem. J.*, **372**, 87-96.
13. De Rycker, M., and Price, C. M. (2004) *Mol. Cell. Biol.*, **24**, 9802-9812.
14. Ham, R. G., and McKeehan, W. L. (1979) *Meth. Enzymol.*, **58**, 44-93.
15. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
16. Wilson, C. M. (1983) *Meth. Enzymol.*, **91**, 236-247.
17. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
18. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Analyt. Chem.*, **68**, 850-858.
19. Rippmann, J. F., Damm, K., and Schnapp, A. (2003) *J. Mol. Biol.*, **325**, 1107.
20. Rippmann, J. F., Damm, K., and Schnapp, A. (2002) *J. Mol. Biol.*, **323**, 217-224.
21. Simonin, F., Hofferer, L., Panzeter, P. L., Muller, S., de Murcia, G., and Althaus, F. R. (1993) *J. Biol. Chem.*, **268**, 13454-13461.