Enzyme Kinetics

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A Colorimetric Substrate for Poly(ADP-Ribose) Polymerase-1, VPARP, and Tankyrase-1**

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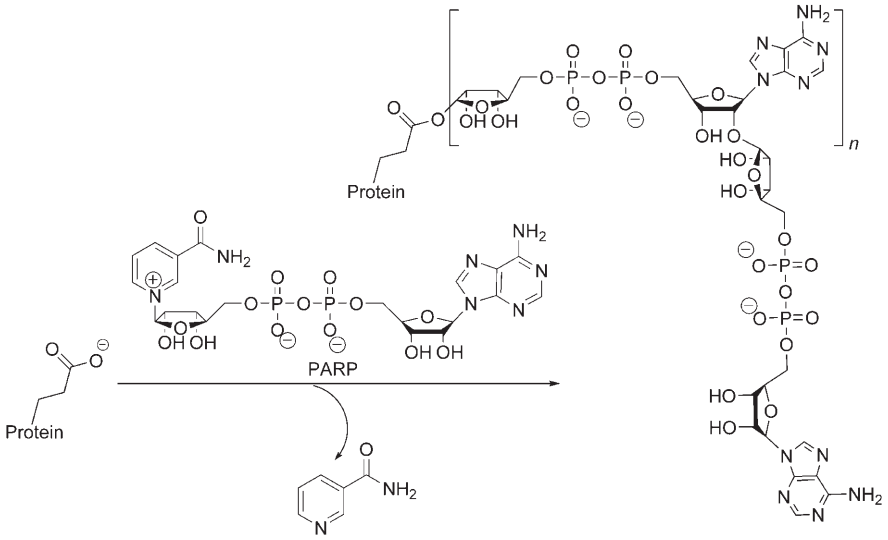
Upon recognition of DNA breaks caused by various genotoxic insults, the enzyme poly(ADP-ribose) polymerase-1 (PARP-1; ADP = adenosine diphosphate) is able to bind damaged DNA and initiate the repair process.[1] Once bound to DNA, PARP-1 is activated and uses β-NAD^+ (NAD^+ = nicotinamide adenine dinucleotide) to poly(ADP-ribosyl)ate proteins such as histones, transcription factors, and itself (in an automodification that leads to inactivation), thus markedly altering the overall size and charge of the modified protein (Scheme 1).[2] Sites for poly(ADP-ribose) (PAR) binding have been identified in numerous DNA-damage checkpoint proteins, including tumor suppressor p53, DNA-ligase III, X-ray repair cross-complementing 1 (XRCC1), DNA-dependent protein kinase (DNA-PK), nuclear factor-κB, and telomerase, and are consistent with the role of PAR in the DNA-repair pathway.[3–5] The rate of PAR synthesis is directly proportional to the number of single- and double-strand breaks found in DNA,[6] and although the levels of PAR may increase more than 100-fold in minutes immediately following DNA damage,[7] synthesis of such polymers is transient and closely regulated by poly(ADP-ribose) glycohydrolase (PARG), an enzyme that cleaves PAR to ADP-ribose monomers.[8]

The cytoprotective role of PARP-1 in response to DNA-damaging agents has been thoroughly examined and is supported by studies with PARP-1-deficient cell lines.[9] Accordingly, inhibition of PARP-1 with small molecules has proven to potentiate anticancer drugs[10,11] and initial studies have demonstrated that some BRCA1-deficient tumor cells (BRCA1 = breast cancer 1, early onset) are quite sensitive to PARP-1 inhibitors.[12,13] On the other hand, extreme DNA damage leads to PARP-1 overactivation and a severe depletion in cellular β-NAD^+/ATP (ATP = adenosine triphosphate) stores. The resulting loss of cellular energy causes necrotic cell death.[14] Thus, overactivation of PARP-1 has a cytotoxic effect, and PARP-1 inhibitors prevent cell death caused by ischemia and injury associated with reactive oxygen species.[15–18]



Scheme 1. Synthesis of poly(ADP-ribose) on glutamic acid residues of protein acceptors as catalyzed by PARP enzymes, producing nicotinamide as a by-product.

PARPs comprise a large family of 18 putative isozymes,[19–21] and although basic enzymatic function and biochemistry has been characterized for at least six members of this family, much work remains to be done in this area. Although PARP-1 accounts for more than 90% of PAR synthesis upon DNA damage,[6,22] it is now known that PAR synthesis by various other PARPs is critical in a variety of cellular processes. Particularly intriguing are the functions of tankyrase-1 and vault PARP (VPARP). Unlike PARP-1, tankyrase-1 is not activated by DNA damage. About 10% of this protein is recruited to telomeres and it has been shown that overexpression of tankyrase-1 results in the lengthening of telomeres through the tankyrase-1-mediated poly(ADP-ribosyl)ation of telomeric repeat binding protein-1 (TRF-1).[23,24] VPARP is most commonly found associated with vault

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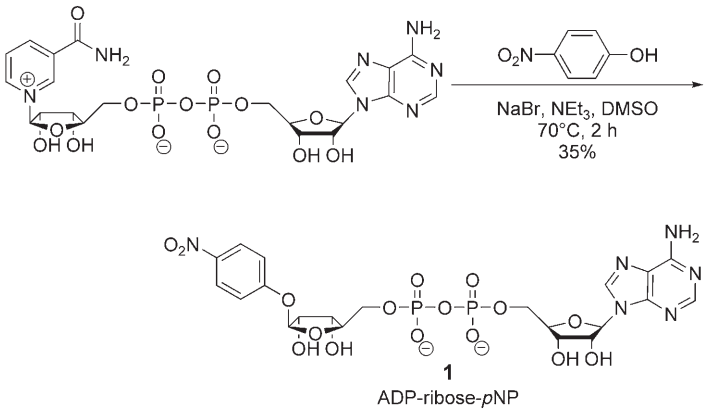
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

particles, but can also localize to the nucleolus, nuclear spindle, or nuclear pores.[25–27]

Even though members of the PARP family have fascinating cellular functions, little progress has been made in developing isozyme-specific PARP inhibitors. This search for potent and selective compounds is hampered by the lack of suitable high-throughput assays. Most commonly, PARP activity is detected with radiolabeled NAD^+,[18,28] but other assays have been developed that employ antibodies,[29] biotinylated NAD^+,[18] or fluorescence-based quantization of NAD^+.[30] Ideally, a PARP assay should be inexpensive, sensitive, rapid, and logistically simple, but methods involving specialized/radioactive reagents can be cost-prohibitive and/or time-consuming when testing a large number of compounds. Herein we report the development of a continuous assay that utilizes a colorimetric PARP substrate, easily synthesized from β-NAD^+, to kinetically monitor PARP-1, tankyrase-1, and VPARP activity.



Scheme 2. Synthesis of PARP substrate from commercially available β-NAD^+ and *p*-nitrophenol.

All PARP isozymes utilize β-NAD^+ to synthesize ADP-ribose polymers, producing nicotinamide as a by-product. Exchanging the nicotinamide moiety of NAD^+ for a colorimetric leaving group would provide a substrate suitable for a continuous kinetic PARP assay. As the use of *p*-nitrophenoxy (*p*NP)-substituted colorimetric substrates has been well-established with various enzyme classes, and as PARP-1 has been known to utilize biotinylated NAD^+ to synthesize poly(ADP-ribose),[31] it seemed that ADP-ribose-*p*NP would make an acceptable colorimetric substrate. The use of commercially available β-NAD^+ as a starting material would greatly simplify the synthesis of this substrate. Very little literature precedent exists for the chemical modification of NAD^+; basic methanolysis is possible and yields a 3.7:1 mixture of β/α anomers,[32] whereas methods employing the NADase/NAD^+ enzymatic system provide *N*- and *O*-(ADP-ribosyl)ation products, albeit in low yield and limited substrate scope.[33–35] As syntheses of cyclic ADP-ribose analogues have utilized nucleophilic metal halides in the presence of triethylamine to successfully and stereoselectively cyclize NAD^+,[36] this strategy was adopted in the preparation of ADP-ribose-*p*NP, **1**. ADP-ribose-*p*NP was synthesized directly from commercially available β-NAD^+ by stirring with sodium bromide and triethylamine in dimethyl sulfoxide (DMSO) at 70 °C for 2 h (Scheme 2). This reaction can easily be performed on the 250–500-mg scale, with isolated product yields of 35 %. Purification involves removal of excess DMSO under reduced pressure and reverse-phase column chromatography. The absolute configuration at the anomeric position was assigned on the basis of coupling constants and NOE experiments, both of which indicate that the product is the β anomer of ADP-ribose-*p*NP (see the Supporting Information). In addition, similar reactions are known to produce products of the β configuration.[36]

Control experiments revealed that ADP-ribose-*p*NP is stable in aqueous buffer solution under PARP assay conditions (50 mM Tris, 10 mM $MgCl_2$, pH 8.0, RT; Tris = tris(hydroxymethyl)aminomethane) for at least 24 h, and is generally stable between pH 4 and 8 (see the Supporting Information). With ADP-ribose-*p*NP in hand, a continuous colorimetric assay for PARP activity was developed and the kinetic parameters for three PARP isozymes were determined. In a 96-well plate, a range of concentrations of ADP-ribose-*p*NP in PARP assay buffer solution were incubated with either PARP-1 (DNase-digested DNA was added to activate PARP-1), tankyrase-1 (refers to "active domain," see the Supporting Information), or VPARP (also refers to the "active domain"), and the optical density at 405 nm was measured every 60 seconds over a time period of 2 h. Changes in absorbance were assessed in triplicate, and blanks containing 0–700 μM ADP-ribose-*p*NP in PARP assay buffer solution were also measured over the same time period. The absorbance of a range of *p*-nitrophenol concentrations was determined at 405 nm, and the slope of this calibration curve (see the Supporting Information) was used to convert the absorbencies to moles of product generated; in this way the kinetic parameters for PARP-1, tankyrase-1, and VPARP were calculated (see Table 1 and Figure 1 a). Consistent with data in the literature, PARP-1 has the largest K_M and V_{max} values (151 μM and 1.30×10^{-3} μmol min^{-1} mg^{-1}, respectively) followed by tankyrase-1 (82 μM and 1.81×10^{-5} μmol min^{-1} mg^{-1}, respectively) and VPARP (46 μM and 2.03×10^{-6} μmol min^{-1} mg^{-1}, respectively). Although exact kinetic parameters for PARP-1 vary with the assay used, for PARP-1, the K_M value with ADP-ribose-*p*NP is consistent with that of the natural β-NAD^+ substrate and the V_{max} value is approximately 100-fold lower with the colorimetric substrate (see Table 1). For tankyrase-1, the K_M value is

Table 1: Comparison of kinetic data for PARP-1, tankyrase-1, and VPARP as reported in the literature and with the ADP-ribose-*p*NP substrate.[a]

	PARP-1	PARP-1 (Refs. [42–44])	tankyrase-1	tankyrase-1 (Ref. [45])	VPARP
k_{cat} [s^{-1}]	0.025	0.41	1.88×10^{-5}	0.71	2.18×10^{-6}
K_M [μM]	151	59–278	82	1500	46
V_{max} [μmol min^{-1} mg^{-1}]	1.30×10^{-3}	0.2–2.4	1.81×10^{-5}	–	2.03×10^{-6}

[a] No literature data is available for the kinetic parameters of VPARP.

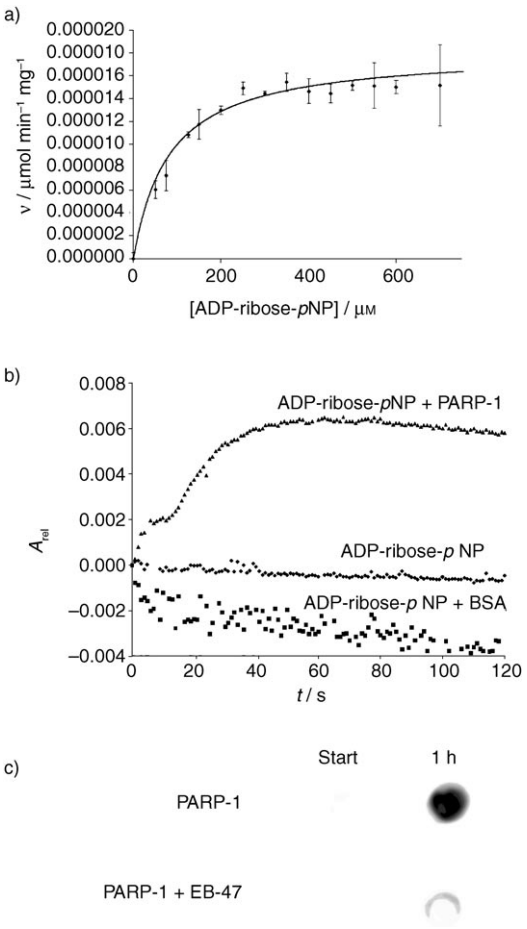


Figure 1. a) Kinetic data for tankyrase-1 obtained by using the ADP-ribose-*p*NP PARP substrate. Analogous curves for PARP-1 and VPARP can be found in the Supporting Information. b) Relative absorbance at 405 nm versus time for 250 μM ADP-ribose-*p*NP, 250 μM ADP-ribose-*p*NP incubated with 20 μM BSA, and 250 μM ADP-ribose-*p*NP incubated with 99 nM PARP-1. All experiments were completed in PARP assay buffer solution with absorbance readings every minute for 2 h. c) Nitrocellulose dot blot illustrating the buildup of PAR after a 1 h incubation of PARP-1, activated DNA, and ADP-ribose-*p*NP. The addition of 100 μM EB-47, a PARP inhibitor, greatly reduces the amount of PAR buildup. PAR levels were assessed with an anti-PAR antibody by using the procedure described in the Supporting Information.

approximately 18-fold lower and the V_{max} value is significantly lower as compared with the values reported in the literature with β-NAD^+ as a substrate. To our knowledge, no data is available on the kinetic parameters of VPARP with β-NAD^+. Control experiments in which bovine serum albumin (BSA) was incubated with the ADP-ribose-*p*NP substrate produced no increase in signal at 405 nm, indicating that this substrate is not processed by proteins in a nonspecific manner (Figure 1b). It has been postulated that PARP-1 is capable of producing PAR from alternative substrates; ADP-ribose chains of no more than four residues in size were observed with 3′-deoxy-NAD^+ as a substrate,[37] and thin-layer chromatography has suggested that PARP-1 can use biotinylated NAD^+ to synthesize PAR as well,[31] although no direct evidence has been presented in the literature. Interestingly, in addition to cleaving the glycosidic bond of ADP-ribose-*p*NP to produce *p*-nitrophenolate, PARP-1 is able to synthesize PAR from the colorimetric substrate. After incubation of PARP-1 with activated DNA and ADP-ribose-*p*NP for one hour, a nitrocellulose dot blot treated with an anti-PAR antibody revealed accumulation of the polymer (Figure 1c). Furthermore, the same experiment completed in the presence of the PARP inhibitor EB-47 (concentration required for 50% inhibition, $IC_{50} = 45$ nM for PARP-1)[38] greatly reduced the amount of PAR formed (Figure 1c).

Next, to demonstrate the potential of this assay to identify isozyme-specific PARP inhibitors, we utilized ADP-ribose-*p*NP to determine the IC_{50} value of the known PARP-1 inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2*H*)-isoquinolinone (DPQ)[39] with PARP-1, tankyrase-1, and VPARP. For these measurements, concentrations of DPQ ranging from 0.05 nM to 10 μM were added to a 96-well plate containing PARP assay buffer solution and ADP-ribose-*p*NP, and the absorbance at 405 nm was measured in triplicate. As shown in Figure 2, DPQ has similar IC_{50} values for all of the

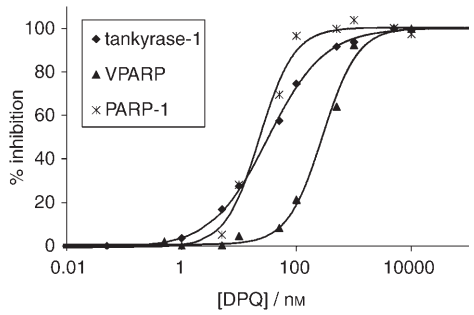


Figure 2. IC_{50} curves for DPQ with PARP-1, tankyrase-1, and VPARP.

PARP isozymes tested, though it has a slightly lower IC_{50} value for PARP-1 (23 nM) and tankyrase-1 (33 nM) as compared with VPARP (281 nM). Literature reports of the IC_{50} value for DPQ with PARP-1 range from 40 nM to 3500 nM.[39–41] Notably, IC_{50} values have never been determined for any compounds with VPARP and tankyrase-1.

Utilizing ADP-ribose-*p*NP as a colorimetric substrate, we have developed a simple, sensitive, and inexpensive kinetic assay for assessing activity of the PARP family of enzymes. This novel substrate has been used to determine the kinetic parameters for PARP-1, tankyrase-1, and VPARP, and it has been employed to obtain IC_{50} values for a small-molecule inhibitor. This type of continuous assay has considerable advantages over standard discontinuous PARP assays. With this new tool for elucidating PARP activity, we now have the ability to gain further understanding of the kinetic activity of the diverse PARP family. As ADP-ribose-*p*NP lends itself easily to milligram-scale synthesis, testing of large libraries to find isozyme-specific inhibitors of these enzymes should be a straightforward task that will provide even more information

about the specific biochemical function of each isozyme and potentially lead to targeted therapies.

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[1] A. Bürkle, *ChemBioChem* **2001**, *2*, 725.
[2] S. Cuzzocrea, Z. Q. Wang, *Pharmacol. Res.* **2005**, *52*, 100.
[3] G. de Murcia, J. Menissier de Murcia, *Trends Biochem. Sci.* **1994**, *19*, 172.
[4] D. D'Amours, S. Desnoyers, I. D'Silva, G. G. Poirier, *Biochem. J.* **1999**, *342*, 249.
[5] W. M. Tong, U. Cortes, Z. Q. Wang, *Biochim. Biophys. Acta* **2001**, *1552*, 27.
[6] M. E. Bonicalzi, J. F. Haince, A. Droit, G. G. Poirer, *Cell. Mol. Life Sci.* **2005**, *62*, 739.
[7] M. L. Meyer-Ficca, R. G. Meyer, D. L. Coyle, E. L. Jacobson, M. K. Jacobson, *Exp. Cell Res.* **2004**, *297*, 521.
[8] L. Davidovic, M. Vodenicharov, E. B. Affar, G. G. Poirier, *Exp. Cell Res.* **2001**, *268*, 7.
[9] F. Le Page, V. Schreiber, C. Dherin, G. De Murcia, S. Boiteux, *J. Biol. Chem.* **2003**, *278*, 18471.
[10] C. A. Delaney, L. Z. Wang, S. Kyle, A. W. White, A. H. Calvert, N. J. Curtin, B. W. Durkacz, Z. Hostomsky, D. R. Newell, *Clin. Cancer Res.* **2000**, *6*, 2860.
[11] P. Jagtap, C. Szabo, *Nat. Rev. Drug Discovery* **2005**, *4*, 421.
[12] H. E. Bryant, N. Schultz, H. D. Thomas, K. M. Parker, D. Flower, E. Lopez, S. Kyle, M. Meuth, N. J. Curtin, T. Helleday, *Nature* **2005**, *434*, 913.
[13] H. Farmer, N. McCabe, C. J. Lord, A. N. Tutt, D. A. Johnson, T. B. Richardson, M. Santarosa, K. J. Dillon, I. Hickson, C. Knights, N. M. Martin, S. P. Jackson, G. C. Smith, A. Ashworth, *Nature* **2005**, *434*, 917.
[14] J. L. Sims, S. K. Berger, N. A. Berger, *Biochemistry* **1983**, *22*, 5188–5194.
[15] I. U. Schraufstatter, P. A. Hyslop, D. B. Hinshaw, R. G. Spragg, L. A. Sklar, C. G. Cochrane, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 4908.
[16] C. Szabo, V. L. Dawson, *Trends Pharmacol. Sci.* **1998**, *19*, 287.
[17] C. Thiemermann, J. Bowes, F. P. Myint, J. R. Vane, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 679.
[18] J. Zhang, S. Lautar, S. Huang, C. Ramsey, A. Cheung, J. H. Li, *Biochem. Biophys. Res. Commun.* **2000**, *278*, 590.
[19] J. C. Ame, C. Spenlehauer, G. de Murcia, *Bioessays* **2004**, *26*, 882.
[20] P. O. Hassa, S. S. Haenni, M. Elser, M. O. Hottiger, *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 789.
[21] S. Smith, *Trends Biochem. Sci.* **2001**, *26*, 174.
[22] J. F. Haince, M. Rouleau, M. J. Hendzel, J. Y. Masson, G. G. Poirier, *Trends Mol. Med.* **2005**, *11*, 456.
[23] S. Smith, I. Giriat, A. Schmitt, T. de Lange, *Science* **1998**, *282*, 1484.
[24] S. Smith, T. de Lange, *Curr. Biol.* **2000**, *10*, 1299.
[25] V. A. Kickhoefer, A. C. Siva, N. L. Kedersha, E. M. Inman, C. Ruland, M. Streuli, L. H. Rome, *J. Cell Biol.* **1999**, *146*, 917.
[26] A. van Zon, M. H. Mossink, M. Schoester, A. B. Houtsmuller, G. L. Scheffer, R. J. Scheper, P. Sonneveld, E. A. Wiemer, *J. Cell Sci.* **2003**, *116*, 4391.
[27] Y. Liu, B. E. Snow, V. A. Kickhoefer, N. Erdmann, W. Zhou, A. Wakeham, M. Gomez, L. H. Rome, *Mol. Cell. Biol.* **2004**, *24*, 5314.
[28] K. J. Dillon, G. C. M. Smith, N. M. B. Martin, *J. Biomol. Screening* **2003**, *8*, 347.
[29] P. Decker, E. A. Miranda, G. de Murcia, S. Muller, *Clin. Cancer Res.* **1999**, *5*, 1169.
[30] K. S. Putt, P. J. Hergenrother, *Anal. Biochem.* **2004**, *326*, 78.
[31] A. Cheung, J. Zhang, *Anal. Biochem.* **2000**, *282*, 24.
[32] R. W. Johnson, T. M. Marschner, N. J. Oppenheimer, *J. Am. Chem. Soc.* **1988**, *110*, 2257.
[33] S. Tono-oka, I. J. Azuma, *J. Heterocycl. Chem.* **1989**, *26*, 339.
[34] S. Tono-oka, I. Azuma, *Liebigs Ann./Recl.* **1997**, 1823.
[35] S. Tono-oka, M. Hatakeyama, *Chem. Pharm. Bull.* **2001**, *49*, 123.
[36] S. Yamada, Q. M. Gu, C. J. Sih, *J. Am. Chem. Soc.* **1994**, *116*, 10787.
[37] R. Alvarez-Gonzalez, *Mol. Cell. Biochem.* **1994**, *138*, 213.
[38] P. Jagtap, G. J. Southan, E. Baloglu, S. Ram, J. G. Mabley, A. Marton, A. Salzman, C. Szabo, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 81.
[39] K. Takahashi, A. A. Pieper, S. E. Croul, J. Zhang, S. H. Snyder, J. H. Greenberg, *Brain Res.* **1999**, *829*, 46.
[40] M. J. Eliasson, K. Sampei, A. S. Mandir, P. D. Hurn, R. J. Traystman, J. Bao, A. Pieper, Z. Q. Wang, T. M. Dawson, S. H. Snyder, V. L. Dawson, *Nat. Med.* **1997**, *3*, 1089.
[41] F. Moroni, E. Meli, F. Peruginelli, A. Chiarugi, A. Cozzi, R. Picca, P. Romagnoli, R. Pellicciari, D. E. Pellegrini-Giampietro, *Cell Death Differ.* **2001**, *8*, 921.
[42] M. Kawaici, K. Ueda, O. Hayaishi, *J. Biol. Chem.* **1981**, *256*, 9483.
[43] S. Beneke, R. Alvarez-Gonzalez, A. Burkle, *Exp. Gerontol.* **2000**, *35*, 989.
[44] M. Banasik, T. Stedeford, R. P. Strosznajder, A. S. Persad, S. Tanaka, K. Ueda, *Acta Neurobiol. Exp.* **2004**, *64*, 467.
[45] J. F. Rippmann, K. Damm, A. Schnapp, *J. Mol. Biol.* **2003**, *325*, 1107.

