Progr Colloid Polym Sci (1997) 106:245–248

A. Goto
H. Hakamata
Y. Kuwahara
R. Goto
P. Walde
P.L. Luisi
T. Imae

Functional nano-structure of aggregates self-organized on the liquid/solid interface–enzymatic polymerization of ADP

Received: 13 October 1996
Accepted: 31 March 1997

Dr. A. Goto (✉)
School of Informatics
University of Shizuoka
52-1 Yada, Shizuoka-shi
Shizuoka-ken, 422, Japan

H. Hakamata · Y. Kuwahara · R. Goto
Graduated School
University of Shizuoka

P. Walde · P.L. Luisi
Polymer Institut in ETH

T. Imae
Fac. Sci. in Nagoya Univ.

Abstract The enzymatic polymerization of ADP was carried out in sodium bis(2-ethylhexyl)sulfosuccinate (AOT)-reversed micellar solutions. The poly (adenylic acid), poly(A), being formed in the water pools precipitated out of the AOT solution together with the enzyme, whose activity was maintained for a long time. The process of the precipitation was studied in comparison with the polymerization in cationic surfactant reversed micelles and the precipitate aggregates were observed by atomic force microscopy (AFM).

Key words Poly(adenylic acid) (poly(A)) – sodium bis(2-ethylhexyl) sulfosuccinate reversed micelle – liquid/solid interface – polynucleotide phosphorylase (PNPase) – atomic force microscopy (AFM)

Introduction

Reversed micellar enzymology has been studied by many researchers [1, 2]. The conversion of apolar compounds by enzymes entrapped in aqueous cores of reversed micelles has drawn the most attention. Reversed micellar enzymology requires not only a large-scale operation, but also enzyme and product recoveries for its practical application. There is still a lack of understanding of many aspects of enzyme behavior in reversed micelles.

We have studied the enzymatic polymerization of ADP in a sodium bis(2-ethylhexyl) sulfosuccinate, AOT, reversed micellar system [3]. This enzymatic polymerization by polynucleotide phosphorylase(PNPase) and Mg^{2+} ion was greatly different from the general aspect of the enzymatic reactions in reversed micellar solutions [3]. This enzymatic reaction in an AOT reversed micellar solution showed that the poly(A), poly(adenylic acid), being formed in the water pools precipitated out together with the enzyme, whose activity was maintained for a long time. The process of the precipitation in AOT-reversed micelles was compared with the polymerization in cationic surfactant-reversed micelles and the precipitated aggregates (PNPase, poly(A) and AOT in various molar fractions) were observed by atomic force microscopy (AFM).

Experimental

AOT and PNPase from *Micrococcus luteus*, commercial products from Sigma were used. Hexadecyltrimethyl

ammonium chloride (HTAC, Merck) was recrystallized in a mixed solvent of MeOH and acetone.

The PNPase-catalyzed synthesis of poly(A) in AOT-reversed micelles was carried out according to the method described in the previous paper [3]. The reaction progress was followed by measuring an unreacted ADP [3]. After a 50 µl of the reaction mixture was added to 1 ml of 1 N $HClO_4$, the solution being centrifuged to precipitate poly(A), the absorbance of unreacted ADP was estimated by measuring the absorbance of a mixture of the supernatant and MeOH (1 : 1) at 260 nm. The synthesis of poly(A) in 0.2 M HTAC/iso-octane : octanol (88 : 12) reversed-micellar solution was carried out in a similar manner to that in the AOT and the reaction progress was measured by the procedure described for AOT with the exception that a 50 µl of the HTAC reaction solution was dissolved in 0.5 ml of 1% *n*-octyl-*β*-D-glucopyranoside solution to prevent an interaction between ADP and HTAC.

HPLC measurement was carried out to detect the produced poly(A) on a Shimazu LC-10A with a nucleogen-DEAE 4000-7 anion exchange column in the same manner as described in the previous paper [3]. After 3 ml of the reaction solution was separated into the supernatant and the precipitate or residue, the solvents being evaporated, 1 ml of ethanol was added to deposit the poly(A). The precipitate was dissolved in 0.5 ml of 50 mM Tris buffer (pH 7) and chloroform was added to remove AOT. After the centrifugation of the solution, the above procedure was repeated. The precipitated poly(A) was dissolved in 100 µl of 10 mM Tris-1 mM EDTA and the aliquot (25 µl) was injected into the HPLC column. The maximum base number for poly(A) which could be analyzed on the column was 30 bases, but poly(A) above that could not be estimated.

The samples for electrophoresis were first prepared according to the procedure of the HPLC sample preparation described above. After the solution was treated with a 1000 µl of ISOGEN solution containing thiocyanate and phenol to remove the PNPase, and with chloroform to remove AOT, 2 ml of 2-propanol was further added to the aqueous phase with the addition of a small amount of glycogen as a coprecipitation agent. The centrifugation and the addition of ethanol were repeated to refine the poly(A). The size of the poly(A) was estimated under 1.2% agarose denaturating gel electrophoresis [4] by using SYBR Green II at 254 nm. RNA Molecular Weight Maker I (7.4, 5.3, 2.8, 1.9, 1.6, 1.0, 0.4, and 0.3 kb) was used. The AFM image of the precipitated aggregate was observed on a Nanoscope III from Digital Instruments using a tapping mode. A mica sheet was set at the bottom of the reaction vial containing 0.2 M AOT/iso-octane reaction solution for 12 h at 37 °C. After its removal, it was soaked in iso-octane for 30 min and then in a mixture of ethanol : water (4 : 1) for 30 min to remove the excess of AOT attached thickly on the mica. The mica was then dried *in vacuo* at room temperature for 2 h. In the case of PNPase, a mica sheet was soaked in an aqueous PNPase solution for 1 min, and the mica was dried under reduced pressure at room temperature for 2 h.

Results and discussion

It is well known that the physical properties of reversed micellar solutions depend significantly upon the molar ratio of water to surfactant, W_0 [5] and the catalytic activity of solubilized enzymes depends also to a greater or lesser extent upon the degree of W_0 [6]. The enzymatic polymerization of ADP was carried out at W_0 of 10, 20, 30 and 40 of 0.3 M AOT/iso-octane reversed micelles at 25 °C. From the reaction mixture, samples were withdrawn at given intervals and the concentration of unreacted ADP was determined, as shown in Fig. 1a, where $[OD]_t/[OD]_0$ (a ratio of OD at any time (t) to OD at the initial time (0)) was plotted against time. From this, it was assumed that the polymerization proceeded the most quickly at $W_0 = 20$. At the end of the polymerization at $W_0 = 10$, 20, and 30, the poly(A) was overwhelmingly found in the precipitate by HPLC, but every supernatant involves scarcely poly(A). The activity of the precipitated PNPase was examined by replacement with a fresh AOT micellar solution containing ADP. Figure 1b shows that since the precipitated PNPase had an activity at $W_0 = 10$, 20 and 30, respectively, but scarcely at $W_0 = 40$, the precipitation of PNPase occurred at $W_0 = 10$, 20 and 30. The polymerization in AOT-reversed micelles seems to need an appropriate size of the water pools and an appropriate ratio of free water to bound water, and the larger the W_0 and the closer it approaches emulsion, the scarcer the tendency to precipitate.

The enzymatic polymerization occurred also in cationic surfactant-reversed micelles of HTAC, as shown in Fig. 2, but it was found by HPLC and electrophoresis that no poly(A) precipitated out of the HTAC micellar solution. Therefore, negative charges of sulfonate group of AOT might play an important role in the precipitation. Gel electrophoresis of poly(A) revealed a broad band on the whole, as shown in Fig. 3. In the case of AOT reversed micelles, the size of poly(A) in the precipitate was in the range 0.6–4.0 kb, and that in the supernatant was in the range 0.4–2.5 kb. In the case of HTAC reversed micelles, no poly(A) was observed in the residue on the bottom of a glass test tube, but the size of poly(A) in the supernatant was in the range 1.0–7.4 kb. The size of poly(A) synthesized in HTAC tended to be larger than that in AOT because the

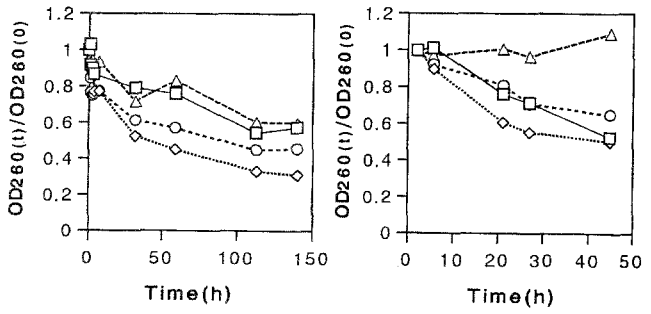

Fig. 1 (a) Effect of W_0 on PNPase catalyzed synthesis of poly(A) in 0.3 M AOT/iso-octane-reversed micelles at 25 °C: $[PNPase]_w$ = 0.76 mg/ml, $[ADP]_w$ = 27.2 mM, $[MgCl_2]_w$ = 10 mM, W_0 = 20 (50 mM Tris-HCl, pH 9.5). (b) Activities of the PNPase in the precipitate at various W_0 at 25 °C. After the above reaction proceeded for 140 h, the supernatant was replaced by a fresh AOT solution containing ADP {$[ADP]_w$ = 14 mM, $[MgCl_2]_w$ = 10 mM, pH 9.5, W_0 = 20 (50 mM Tris-HCl)}. The arrow shows the replacement of a fresh AOT solution; (□) W_0 = 10, (◇) W_0 = 20, (○) W_0 = 30, (△) W_0 = 40

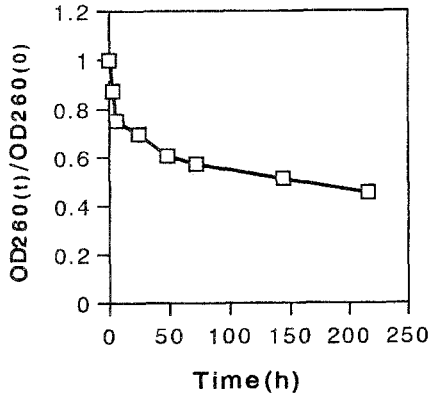

Fig. 2 PNPase-catalyzed synthesis of poly(A) in 0.2 M HTAC/iso-octane/octanol-reversed micelles at 25 °C: $[PNPase]_w$ = 0.83 mg/ml, $[ADP]_w$ = 11 mM, $[MgCl_2]_w$ = 10 mM, W_0 = 20 (50 mM Tris-HCl, pH 9.5)

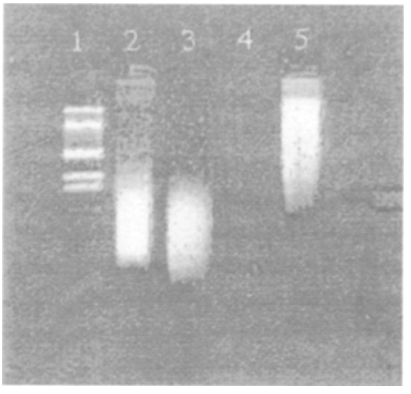

Fig. 3 Gel electrophoretic pattern of poly(A) formed in AOT and HTAC micellar solutions: PNPase-catalyzed synthesis of poly(A) was carried out in 0.2 M AOT or HTAC reversed micelles of 5 ml at 25 °C: $[PNPase]_w$ = 1 mg/ml, $[ADP]_w$ = 20 mM, $[MgCl_2]_w$ = 10 mM, W_0 = 20 (50 mM Tris-HCl, pH 9.5). After the polymerizations proceeded for 24 h (the yields were 70% for AOT and 60% for HTAC), the respective reaction solutions were separated into the supernatant and the precipitate, and they were treated according to the experiment. The purified poly(A) was dissolved in 50 µl of 10 mM Tris buffer (pH 7), was diluted 10 times, and the constant volume was injected. (precipitate: 1 µl for AOT and 10 µl for HTAC; supernatant; 10 µl for AOT and 0.5 µl for HTAC) 1: RNA Marker, 2: AOT (precipitate), 3: AOT (supernatant), 4: HTAC (precipitate), 5: HTAC (supernatant)

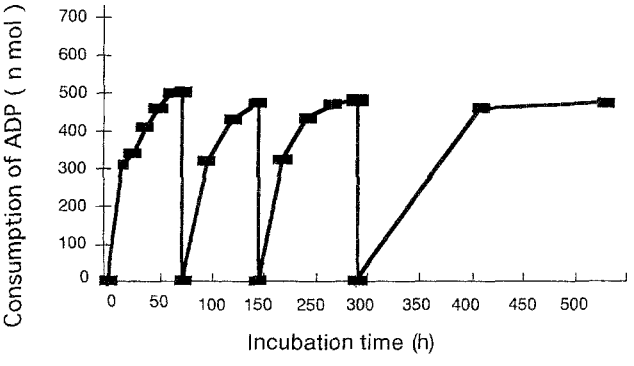

Fig. 4 Activities of PNPase on the interface of glass/0.2 M AOT-reversed micellar solution. After the reaction (the total volume of 0.2 M AOT solution, 3 ml, $[PNPase]_w$ = 1 mg/ml, $[ADP]_w$ = 10 mM, $[MgCl_2]_w$ = 10 mM, W_0 = 20 [50 mM Tris-HCl, pH 9.5]) proceeded for a definite time, the supernatant was removed and the precipitate was rinsed three times with AOT solutions containing buffer (W_0 = 20) without ADP, and a fresh AOT solution containing ADP ($[ADP]_w$ = 10 mM, $[MgCl_2]_w$ = 10 mM, W_0 = 20 [50 mM Tris-HCl, pH 9.5]) was added to the precipitate. The arrows show an addition of a fresh AOT solution. The ADP was determined from the absorbance at 259 nm ($\varepsilon_{max} = 15.4 \times 10^3$)

polymerization proceeds on the interface of the water pool due to the electric attraction between the positive HTAC monolayer surrounding the water pool, and negative poly(A).

The addition of a fresh AOT-reversed micellar solution containing ADP to the precipitate resulted in a new start to the reaction, showing that the PNPase precipitated with the poly(A), as shown in Fig. 1b. Interestingly, each repeated addition of a fresh reversed micellar solution to the precipitates resulted in a new polymerization of ADP. The activity of the PNPase on the interface of the glass surface/0.2 M AOT solution was then examined, as shown in Fig. 4. The ca. 500 nmol of ADP was consumed per 72 µg PNPase, in which 720 nmol of the ADP was contained as a substrate in 1 ml of 0.2 M AOT reaction solution. This revealed that the yield was maintained to be constantly ca. 70% for 3 weeks, and poly(A) was successively accumulated on the glass surface. Therefore, it is reasonable to consider that the precipitate corresponds to functional aggregates.

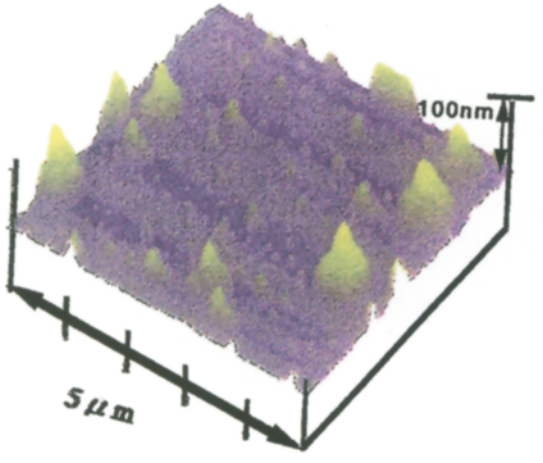

Fig. 5 AFM image of PNPase-catalyzed synthesis of poly(A) on the solid surface. A mica sheet was soaked in 0.2 M AOT/iso-octane solution ($[PNPase]_w = 1$ mg/ml, $[ADP]_w = 10$ mM, $[MgCl_2]_w = 10$ mM, $W_0 = 20$ [50 mM Tris-HCl, pH 9.5]) at 37 °C for 12 h

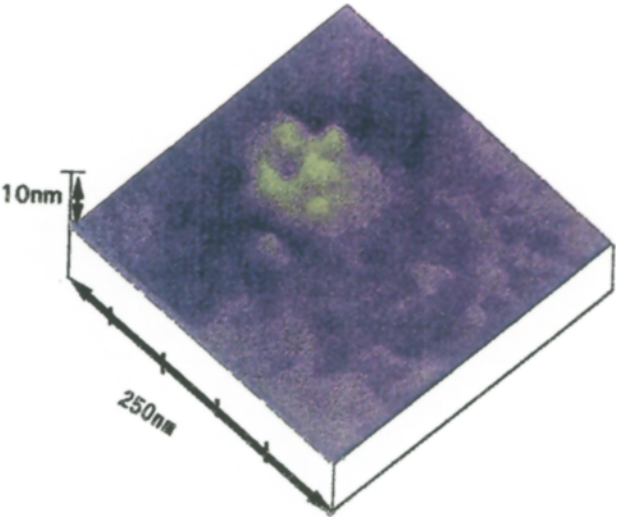

Fig. 6 AFM image of PNPases. [PNPase] = 0.1 mg/ml (50 mM Tris-HCl, pH 9.5)

The nonostructure of the functional precipitate was studied by AFM. Figure 5 shows the AFM image of the precipitated product. Large particles with sizes 200–500 nm and heights of 100 nm were observed, but the control picture scarcely showed such particles (data not shown). Such large particles must be covered thickly with AOT layers because they can be observed by rinsing of the AOT. The PNPase dissolved in the buffer was observed by AFM. As shown in Fig. 6, the AFM image of PNPase molecules in the absence of ADP shows several masses, of which a unit corresponds to be ca. 20 nm. The unit can be assumed to be a trimer of PNPase because it is known that PNPases forms a trimer [7]. Since the 85 Å [8] PNPase molecules in the absence of ADP were found by electron microscopy, the size in the AFM image seems to be larger. One of the factors is considered; the PNPase sample for AFM seems to be more hydrated compared with that for electron microscopy. The above result shows that the PNPases are much smaller than the precipitated articles in Fig. 5, and the PNPases and the poly(A) which form the particles could not be discriminated by AFM.

It may be concluded that the particles in the precipitate observed by AFM are nano-structured functional aggregates which are self-organized on the interface between the solid phase of glass and the liquid phase of AOT-reversed micellar solution. Our results show not only a novel synthesis of biopolymer on the interface of solid/oil liquid, but also a possibility of enzyme and product recovery which can lead to a large-scale application procedure.

References

1. Luisi PL, Gimini M, Pileni MP, Robinson H (1988) Biochim Biophys Acta 7:209–246
2. Hilhorst R (1989) In: Pileni MP (ed) Structure and Reactivity in Reverse Micelles Elsevier p 323–341
3. Walde P, Goto A, Monnard P-A, Wessicken MJ, Luisi PL (1994), J Am Chem Soc 116:7541–7547
4. Lehrach H, Diamon D, Wozney JM, Boedteker H (1977) Biochemistry 16:4743–4751
5. Zulauf M, Eicke H-F (1979) J Phys Chem 83:480–486. Hauser H, Hearing G, Pande A, Luisi PL (1989) J Phys Chem 93:7869–7876
6. Martinek K, Levashov AV, Khmelnitsky YuL, Klyachko NL, Berezin LV (1986) Eur J Biochem 155:453–468
7. Barbehenn EK, Craine JE, Chrambach A, Klee CB (1982) J Biol Chem 257: 1007–1016
8. Valentine RC, Thang MN, Grunberg-Manago M (1969) J Mol Biol 39: 389–391