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Disaggregation of Platelet Aggregates Formed by the Action of Thrombin in the Presence of Hydrogen Peroxide

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Abstract—The aggregation and change in the intracellular Ca^{2+} concentration induced by thrombin (0.005-0.22 U/ml) in the presence of H_2O_2 (0.05-0.6 mM) was investigated. Under the chosen experimental conditions (incubation time of platelets with H_2O_2 not more than 15 sec), H_2O_2 neither accelerated nor inhibited the thrombin-induced platelet aggregation. However, platelet aggregates formed by the action of thrombin in the presence of H_2O_2 were unstable and disaggregated. Disaggregation was abolished by catalase added after thrombin. The disaggregation effect was dose-dependent; the process of disaggregation was confirmed by electron microscopy. Hydrogen peroxide did not influence thrombin-induced increase in the intracellular Ca^{2+} concentration, but dose-dependently accelerated Ca^{2+} extrusion from the platelet cytoplasm.

Key words: hydrogen peroxide, platelet disaggregation, thrombin, Quin 2, intracellular Ca^{2+}

Platelets are blood cells that play an important role in hemostasis; their functional activity changes by the action of aggregation agonists and inhibitors. Aggregation agents such as ADP and thrombin activate the signal systems of secondary messengers resulting in increase in the intracellular Ca^{2+} concentration, and exhibit receptor-mediated inhibition of the adenylate cyclase activity. Formation of the aggregation receptors $\alpha_{IIb}\beta_3$ on the plasmatic membrane and exposition of P-selectins due to which the intercellular contacts and platelet aggregates are formed occur with participation of intracellular Ca^{2+} and calcium-activated systems [1, 2]. Platelet disaggregation is studied to a lesser extent than aggregation. It is known that ADP at low concentrations causes formation of aggregates, which then disaggregate spontaneously [3]. cGMP was shown to play an important role in the mechanisms of platelet disaggregation [4]. ADP at high concentrations and thrombin cause irreversible platelet aggregation. As shown by us earlier, the irreversible ADP-induced platelet aggregation becomes reversible in the presence of H_2O_2 [5]. It is known that the effect of H_2O_2 on the functional activity of platelets is ambiguous: H_2O_2 at high concentrations causes platelet aggregation, but at low concentrations inhibits the platelet aggregation capacity [6, 7]. Based on the data on thrombin-induced platelet aggregation and change in the intracellular Ca^{2+} concentration in the presence of H_2O_2, we conclude that along with the aggregatory and inhibitory properties, H_2O_2 also exhibits disaggregation capacity.

MATERIALS AND METHODS

To obtain the platelet suspension, the cell pellet was washed twice as described in [8]. To study cell aggregation, the platelets were suspended in 13.3 mM Tris-buffer, pH 6.5, containing EDTA (120 mM NaCl, 15.4 mM KCl, 1.5 mM EDTA, and 6 mM D-glucose). To determine the cytoplasmic Ca^{2+} concentration, the platelets were suspended in 10 mM Mops-buffer (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5 mM D-glucose, and 0.1% BSA), pH 7.4, not containing $CaCl_2$ and $MgCl_2$. The platelet suspensions (concentration $2.5 \cdot 10^9$ cells per ml of buffer) were stored at room temperature as the initial experimental suspensions.

To study the platelet aggregation and disaggregation, 50 μl of the initial platelet suspension in Tris-buffer was placed in a cuvette of an aggregometer containing 420 μl of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, 1 mM $CaCl_2$), pH 7.35, containing Ca^{2+}. To inhibit activity of the enzymes decomposing H_2O_2, 10 μl of 500 μM NaN_3 was

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added (final concentration 10 μM), the mixture was incubated with stirring for 2 min at 37°C, and then 10 μl of H_2O_2 and 10 μl of thrombin at various concentrations were added. Control experiments were performed in the absence of H_2O_2. The aggregation rate (v, % of light transmission per 1 min) was determined automatically from the aggregation curve. The degree of disaggregation was characterized by an α parameter determined as a ratio $\alpha = [(T_1 - T_2)/T_1]\cdot 100\%$, where T_1 is the maximal value of light transmission of the platelet suspension on aggregation and T_2 is the minimal value of light transmission of the platelet suspension on disaggregation determined for 10 min.

The intracellular Ca^{2+} concentration in platelets was determined using a fluorescent probe Quin 2 as described in [9]. Kinetics of change in intensity of intracellular Quin 2 fluorescence was monitored at 495 nm (λ_{ex} = 339 nm) with stirring at 37°C. The concentration of cytoplasmic calcium was calculated according to [10].

The study of platelet aggregation and spectrofluorimetric experiments were performed using an AP2110 analyzer of platelet aggregation and a LSF 1211A spectrofluorimeter from Scientific-Production Center SOLAR (Belarus). The number of platelets was determined using a Goryaev chamber.

For electron microscopy, the material was fixed according to the ordinary procedure [11]. The samples were placed in a epon–araldite mixture. Ultrafine slices were obtained using a LKB microtome (Sweden) and then studied and photographed using a JEM-100CX electron microscope from JEOL (Japan).

The results are presented as average values ± the standard deviation calculated for five independent experiments.

Reagents. In this study we used 3-[N-morpholino] propanesulfonic acid (MOPS) and ionomycin from Sigma (Germany); tris(hydroxymethyl)aminomethane (Tris) from Reanal (Hungary); acetoxymethyl ester of Quin 2 from Calbiochem (Germany); bovine thrombin with activity 2.75 NIH U/mg protein from Kaunas Bacterial Preparations Production (Lithuania); sodium azide from Fluka (Switzerland). Other reagents were produced by Reakhim (Russia) and Belmedpreparaty (Belarus).

RESULTS

Thrombin-induced platelet aggregation in the absence and in the presence of 0.4 mM H_2O_2 is presented in Fig. 1. In the absence of H_2O_2, thrombin induced irreversible aggregation (Fig. 1, curve *1*). In the presence of H_2O_2 thrombin-induced aggregation became reversible (Fig. 1, curve *2*), indicating disaggregation of the earlier formed aggregates. H_2O_2 (0.4 mM) in the absence of thrombin did not cause changes in light transmission of the cell suspension (Fig. 1, curve *4*). Catalase (1000 U/ml), added 1 min after thrombin, abolished disaggregation (Fig. 1, curve *3*). Under the experimental conditions shown in Fig. 1, thrombin was added 15 sec after addition of H_2O_2. Under such conditions thrombin induced platelet aggregation up to the same maximal level as in the absence of H_2O_2.

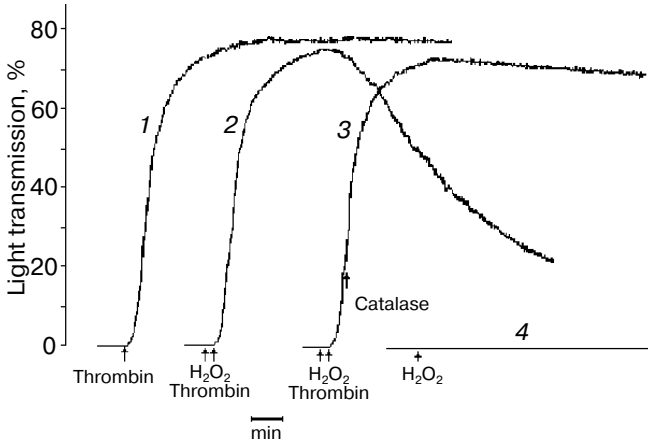

Fig. 1. Thrombin-induced platelet aggregation in the absence (*1*) and in the presence of H_2O_2 (*2*) and effect of catalase (*3*); *4*) H_2O_2 action in the absence of thrombin. Concentrations: thrombin, 0.06 U/ml; catalase, 1000 U/ml; H_2O_2, 0.4 mM.

Figure 2 presents the quantitative analysis of data on single platelets at various stages of thrombin-induced aggregation in the presence of H_2O_2. In the samples taken at the stage of the maximal aggregation, the number of single platelets was 10 times less than that in the initial suspension. In the samples taken at the stage of reverse aggregation, the number of single platelets increased by 60% compared with the initial suspension.

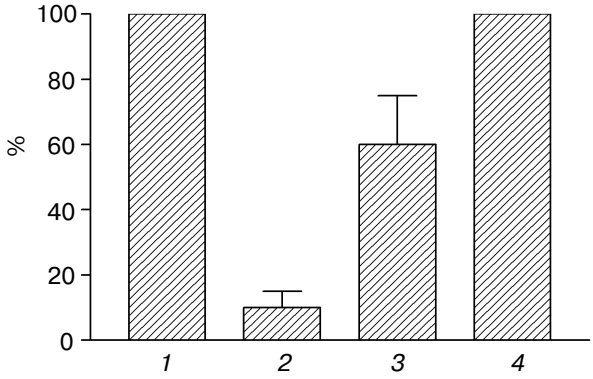

Fig. 2. Change in the number of single platelets: *2*) at the stage of the maximal aggregation; *3*) after disaggregation; *4*) under the influence of H_2O_2 in the absence of thrombin. The number of platelets in the initial suspension was taken as 100% (*1*). Concentrations: thrombin, 0.06 U/ml; H_2O_2, 0.4 mM.

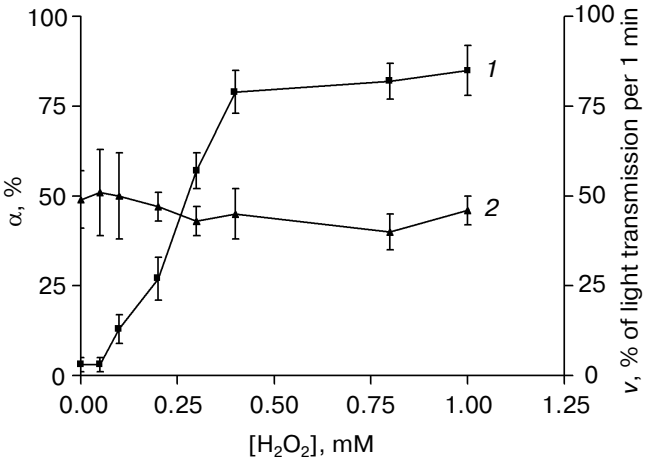

Fig. 3. Degree of disaggregation (*1*) and the rate of thrombin-induced aggregation (*2*) versus H_2O_2 concentration. Thrombin, 0.06 U/ml.

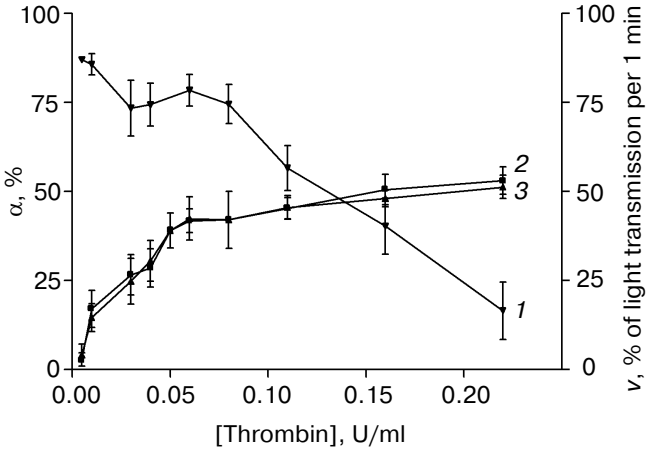

Fig. 4. Degree of disaggregation (*1*) and the rate of platelet aggregation (*2*) in the presence of 0.4 mM H_2O_2 versus thrombin concentration; *3*) rate of platelet aggregation in the absence of H_2O_2.

The platelet concentration did not change by the action of H_2O_2 in the absence of thrombin. The former also did not change on the more prolonged incubation (5-20 min) with H_2O_2 (data not presented here), indicating that 0.4 mM H_2O_2 does not cause aggregate formation or cell destruction.

The degree of disaggregation depended on H_2O_2 concentration (Fig. 3). The degree of disaggregation was 50% of the maximal one at 0.25 mM H_2O_2. Disaggregation was 78 ± 6% at 0.4 mM H_2O_2 and remained at the same level on increase in H_2O_2 concentration to 1 mM. The data indicate that H_2O_2 at concentrations 0.1-1.0 mM did not affect the rate of thrombin-induced platelet aggregation. Consequently, under the chosen experimental conditions (the incubation time of platelets with H_2O_2 not more than 15 sec) H_2O_2 neither accelerated nor inhibited the thrombin-induced platelet aggregation. However, the platelet aggregates formed in the presence of H_2O_2 were unstable and disaggregated.

The disaggregating effect of 0.4 mM H_2O_2 on the thrombin-induced platelet aggregation was different at various thrombin concentrations (Fig. 4). Thus, in the presence of 0.4 mM H_2O_2, the degree of disaggregation of aggregates formed by the action of 0.04-0.08 U/ml thrombin was about 75-80% and that of aggregates formed by the action of 0.16 and 0.22 U/ml thrombin – 40 and 17%, respectively. So, reversibility of the thrombin-induced platelet aggregation in the presence of H_2O_2 decreased on increase in thrombin concentration. As shown in Fig. 4, the rate of thrombin-induced aggregation did not change in the presence of H_2O_2. This fact indicates that H_2O_2 did not influence the aggregative capacity of thrombin.

As shown by electron microscopy (Fig. 5), the aggregates are present in the samples fixed at the stage of the maximal light transmission of the studied suspension, while single platelets are present in the samples fixed at the stage of the minimal light transmission. The data confirm that the platelet aggregates formed by the action of thrombin in the presence of H_2O_2 are unstable and decompose to single platelets, which means platelet dis-

Thrombin-induced change in the intracellular Ca^{2+} concentration in the presence of various H_2O_2 concentrations

H_2O_2 concentration, mM	Intracellular Ca^{2+} concentration, nM		
	before the action of thrombin	after addition of thrombin (0.06 U/ml)	
		in 0.6 min (maximal value)	in 5 min
0	94 ± 6	355 ± 47	299 ± 22
0.01	92 ± 5	350 ± 35	226 ± 13
0.04	92 ± 8	317 ± 42	122 ± 13
0.06	94 ± 10	330 ± 31	105 ± 15
0.13	90 ± 8	336 ± 16	107 ± 15
0.3	88 ± 8	327 ± 35	106 ± 5
0.4	91 ± 9	367 ± 28	89 ± 8
0.6	96 ± 7	346 ± 44	92 ± 12
1	96 ± 5	360 ± 36	95 ± 10

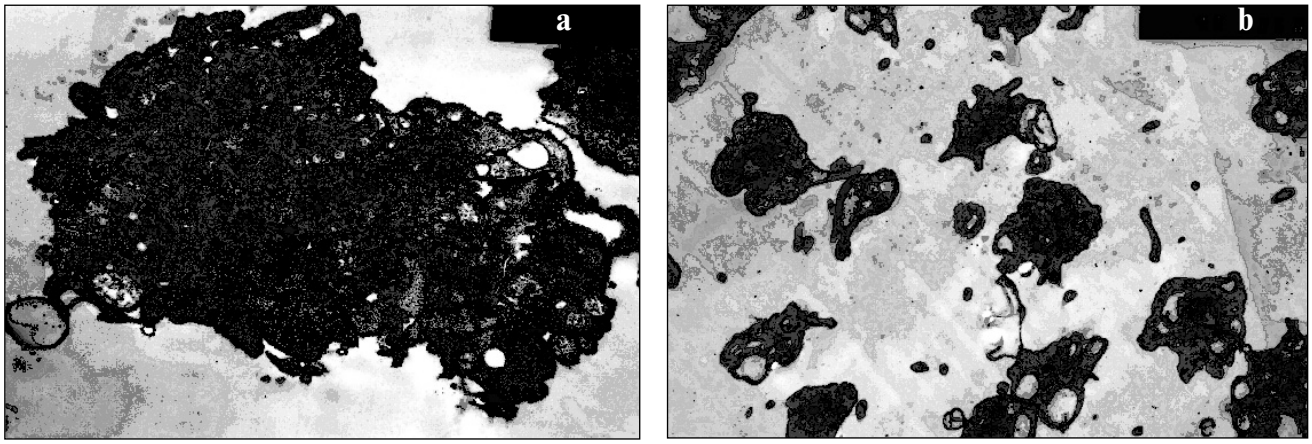

Fig. 5. Platelets fixed at the stage of the maximal (a) and minimal (b) light transmission on thrombin-induced aggregation in the presence of H_2O_2. Magnification: a) ×6000; b) ×4000. Concentrations: thrombin, 0.06 U/ml; H_2O_2, 0.4 mM.

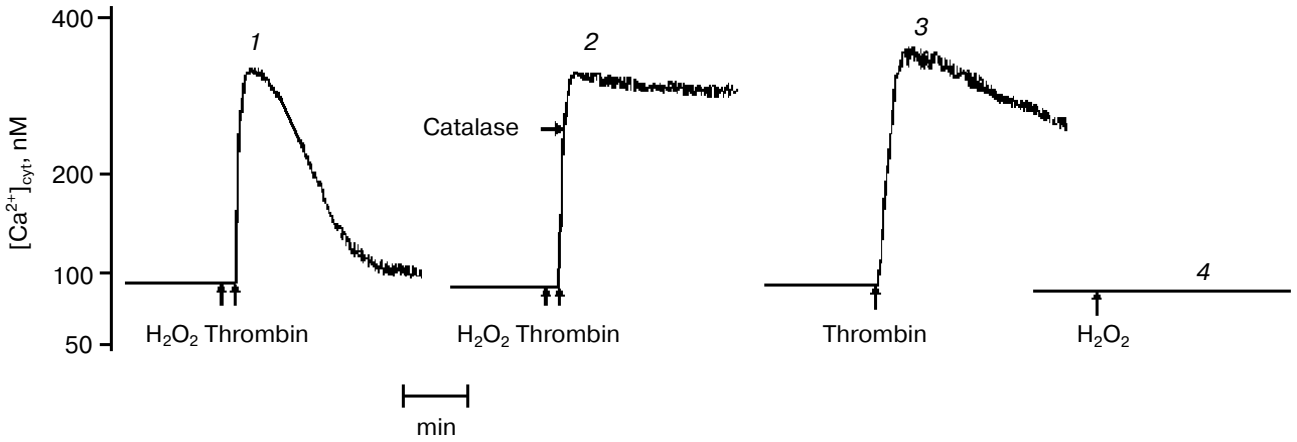

Fig. 6. Thrombin-induced change in the intracellular Ca^{2+} concentration in the presence (*1*) and in the absence of H_2O_2 (*3*) and the effect of catalase (*2*); *4*) effect of H_2O_2 in the absence of thrombin. Concentrations: thrombin, 0.06 U/ml; catalase, 1000 U/ml; H_2O_2, 0.4 mM.

aggregation. Disaggregated platelets had irregular shape with pseudopodia and empty granules (Fig. 5b).

Data on the effect of H_2O_2 on the thrombin-induced change in the intracellular Ca^{2+} concentration are presented in Fig. 6 and the table. In the presence of 0.4 mM H_2O_2 the level of cytoplasmic calcium, increased by the action of thrombin, decreased more rapidly than in the absence of H_2O_2 (Fig. 6, curves *1* and *3*). Catalase abolished the effect of H_2O_2 (Fig. 6, curve *2*). In the presence of various H_2O_2 concentrations, thrombin increased the intracellular calcium to almost the same level (table). The dependence of the cytoplasmic calcium level on H_2O_2 concentration was observed 5 min after addition of thrombin, and in the presence of 0.4 mM H_2O_2 it decreased to the basal level. H_2O_2 itself did not change the basal Ca^{2+} level. The data indicate that H_2O_2 accelerates the process of Ca^{2+} removal from the cytoplasm.

DISCUSSION

Thrombin is a powerful aggregation inducer since it causes irreversible platelet aggregation. As shown in this study, unstable platelet aggregates are formed by the action of thrombin in the presence of H_2O_2; then they disaggregate. It is known that H_2O_2 at concentrations higher than 2 mM causes platelet aggregation [6], but at concentrations lower than 1 mM, it inhibits aggregation induced by various aggregants [7]. In this work, we studied the action of 0.05-0.6 mM H_2O_2. At these concentrations and under the chosen experimental conditions (incubation time with platelets not more than 15 sec), H_2O_2 did not influence the platelet light transmission, the number of cells in the initial suspension, the rate of thrombin-induced platelet aggregation, and thrombin-induced increase in cytoplasmic Ca^{2+} concentrations,

and consequently, did not cause any toxic, aggregatory, or inhibitory action. Nonetheless, the effect of H_2O_2 is obvious, since disaggregation was dose-dependent, which was proven by electron microscopy.

The properties of thrombin can change on its oxidation. As shown in [12], thrombin oxidized by hypochlorite or the myeloperoxidase–H_2O_2–Cl^- system exhibited a lesser aggregatory activity than the non-oxidized one. However, it is also shown in the same paper that 0.2 mM H_2O_2 by itself is not able to oxidize thrombin. Our results indicating that aggregation and increase in the intracellular Ca^{2+} concentration induced by thrombin did not change in the presence of H_2O_2 agree well with the data [12] that H_2O_2 does not affect the aggregatory activity of thrombin.

The data indicate that calcium extrusion from the platelet cytoplasm is accelerated in the presence of H_2O_2. Catalase abolished this effect of H_2O_2; this indicates participation of exogenous H_2O_2 in activation of Ca^{2+}-transporting systems. In the platelet plasmatic membranes Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger are the systems removing intracellular calcium mobilized by aggregants from the cytoplasm [13, 14]. For various cell types, it has been shown that incubation with H_2O_2 results in stimulation of Na^+-Ca^{2+} exchange [15] and cGMP-mediated change in the activity of Ca^{2+}-ATPase of the plasmatic membrane [16]. However, it is known that $\alpha_{IIb}\beta_3$ aggregation receptors contain SH-groups, which can also be a target for H_2O_2 [17]. It cannot be excluded that their modification can result in activation of the transport systems decreasing the intracellular Ca^{2+} concentration.

Our results show that removal of Ca^{2+} from the cytoplasm of thrombin-activated platelets can cause platelet disaggregation.

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REFERENCES

1. Parise, L. V. (1999) *Curr. Opin. Cell Biol.*, **11**, 597-601.
2. Merten, M., and Thiagarajan, P. (2000) *Circulation*, **102**, 1931-1936.
3. Maayani, S., Tagliente, T. M., Schwarz, T., Martinelli, G., Martinez, R., and Shore-Lesserson, L. (2001) *Platelets*, **12**, 83-93.
4. Severina, I. S. (1994) *Biochemistry (Moscow)*, **59**, 231-240.
5. Samal, A. B., Cherenkevich, S. N., and Khmara, N. F. (1990) *Biokhimiya*, **55**, 786-790.
6. Irani, K., Pham, Y., Coleman, L. D., Roos, C., Cooke, G. E., Miodovnik, A., Karim, N., Wilhide, C. C., Bray, P. F., and Goldschmidt-Clermont, P. J. (1998) *Arterioscler. Thromb. Vasc. Biol.*, **18**, 1698-1706.
7. Belisario, M. A., Tafuri, S., Domenico, C. D., Squillacioti, C., Morte, R. D., Lucisano, A., and Staiano, N. (2000) *Biochim. Biophys. Acta*, **1495**, 183-193.
8. Samal, A. B., and Loiko, E. N. (2000) *Biochemistry (Moscow)*, **65**, 230-236.
9. Samal, A. B., Timoshenko, A. V., Loiko, E. N., Kaltner, H., and Habius, H.-J. (1998) *Biochemistry (Moscow)*, **63**, 516-522.
10. Hallam, T. J., Sanchez, A., and Rink, T. J. (1984) *Biochem. J.*, **218**, 819-827.
11. Bogolepov, N. N. (1976) *Methods of Electronic Microscopy Studies of Brain* [in Russian], Meditsina, Moscow.
12. De Cristofaro, R., and Landolfi, R. (2000) *Thromb. Haemost.*, **83**, 253-261.
13. Martin, V., Bredoux, R., Corvazier, E., Papp, B., and Enouf, J. (2000) *Hypertension*, **35**, 91-102.
14. Valant, P. A., Adjei, P. N., and Haynes, D. H. (1992) *J. Membr. Biol.*, **130**, 63-82.
15. Reeves, J. P., Bailey, C. A., and Hale, C. C. (1986) *J. Biol. Chem.*, **261**, 4948-4955.
16. Johansson, J. S., and Haynes, D. H. (1992) *Biochim. Biophys. Acta*, **1105**, 40-50.
17. Yan, B., and Smith, J. W. (2000) *J. Biol. Chem.*, **275**, 39964-39972.