Carbohydrate Research, 217 (1991) 213–220
Elsevier Science Publishers B.V., Amsterdam

Determination of kinetic parameters for maltotriose and higher malto-oligosaccharides in the reactions catalyzed by α-D-glucan phosphorylase from potato

Toshihiko Suganuma*, Jun-Ichiro Kitazono, Kazuhiro Yoshinaga, Shigeo Fujimoto, and Tomonori Nagahama

Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Kagoshima 890 (Japan)

(Received September 5th, 1990; accepted for publication January 4th, 1991)

ABSTRACT

For kinetic studies on its synthetic and phosphorolytic reactions, α-D-glucan phosphorylase from potatoes was purified chromatographically until free of D-enzyme. Purified maltotriose (G_3) is a poor primer in the phosphorylase-catalyzed synthetic reaction, showing an anomalous time course and making previous attemps to determine its kinetic parameters unsuccessful. In the present work the true rate of the G_3-primed reaction was obtained from linear plots obtained by incorporating a sufficient quantity of β-amylase in the digest to eliminate the more rapidly reacting G_4 formed from the G_3. A K_m value of 9.4 ± 0.8 mM for G_3 was calculated from the data by a nonlinear least-squares method. Kinetic parameters for a series of higher malto-oligosaccharides (G_4–G_8) were also determined in both the synthetic and the phosphorolytic directions. A large change in the values of K_m and V/e was seen on going from G_3 to G_4 for the synthetic reaction, and from G_4 to G_5 for the phosphorolytic. For the higher saccharides the V/e values do not vary strongly with increasing d.p., while the K_m values tend to decrease, as has seen in the reactions of other plant phosphorylases.

INTRODUCTION

α-Glucan phophorylase (EC 2.4.1.1) belongs to a group of vitamin B_6 enzymes having a novel catalytic mechanism that involves the participation of the phosphate group of pyridoxal 5′-phosphate (PLP)[1–3]. This mechanism is common to both plant and animal phosphorylase, but the plant enzyme differs from the animal in amino acid sequence, the presence of a glycogen storage site, regulation mechanism, *etc.*[4–6]. Phosphorylase catalyzes both directions of the reaction shown in Eq. *1*, where the G-1-P represents glucose 1-phosphate and P_i inorganic phosphate.

$$G_n + \text{G-1-P} \rightleftharpoons G_{n+1} + P_i \qquad (1)$$

In the synthetic reaction, the malto-oligosaccharide cosubstrate G_n is called "primer", with n having a reported minimum value of three[7]. Although there have been

* To whom correspondence should be addressed.

0008-6215/91/$ 03.50

several reports[8–14] on the ability of maltotriose (G_3) to serve as a primer, the kinetic parameters of the reaction with this substrate had not been determined. This was mainly due to the unusual kinetics that result when purified G_3 is used as the primer: the time course of the reaction is a nonlinear, accelerating curve.

In the present study, we were able to measure the true rate of the G_3-primed reaction by including β-amylase in the incubation mixture. We determined the kinetic parameters of potato phosphorylase action in both the synthetic and phosphorolytic directions, using maltotriose as well as higher malto-oligosaccharides as substrates.

RESULTS AND DISCUSSION

Maltotriose-primed reaction of potato phosphorylase. — There are three problems to be solved for determining the kinetic parameters for G_3: The first is higher-saccharide contamination of commercially obtainable G_3; the second is D-enzyme contamination of the phosphorylase preparation[9,11]; and the third is the anomalous time course of the G_3-primed reaction as shown in Fig. 1.

Commercial maltotriose was usually contaminated with other (probably higher) saccharides, and the time course of the reaction run with impure G_3, differed from that with purified substrate in showing a lag phase in the initial stage of the reaction. Thus, the saccharide was purified on a carbon column[15] before use. The same purification procedure was also used to obtain primer-free G-1-P from the commercial sodium salt (Nacalai Tesque, Inc.).

It seemed possible that the phosphorylase preparation would be contaminated by

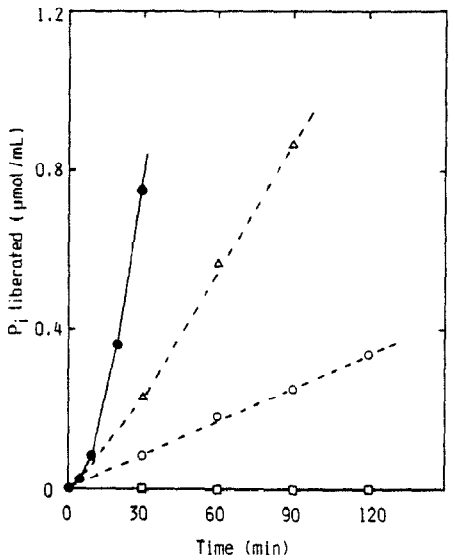

Fig. 1. Time course of the G_3-primed reaction of potato phosphorylase in the presence and in the absence of β-amylase: △, with β-amylase, 0.4 unit/mL; ○, with β-amylase, 4 units/mL; ●, without β-amylase; □, without primer (G_3).

(1→4)-α-glucanohydrolases or D-enzyme[11]. The former enzymes can degrade glucan primer in the phosphorylase digest. The latter enzyme could seriously affect the G_3-primed reaction of phosphorylase because it converts G_3 into G_5 + G by transfer action[16,17], and thus increases the apparent rate of the G_3 reaction[11].

Potato enzyme was purified essentially according to Kamogawa *et al.*[18]. We used a column of DEAE-Toyopearl instead of DEAE-Sephadex, because the former column was able to separate the phosphorylase from D-enzyme more effectively than the latter. Fig. 2 presents an example of chromatography on DEAE-Toyopearl for the final purification. D-Enzyme activity was assayed by measuring the higher saccharides formed from G_3, using phosphorylase as a secondary enzyme (see Experimental). This assay is more specific than the method based on the measurement of glucose[11,19], since glucose-forming enzymes such as α-glucosidase would also be detected by the latter. The enzyme preparation was proved on long-term incubations to be free of the activities of D-enzyme, α-glucosidase, and amylase, with G_3, *p*-nitrophenyl α-glucoside, and soluble starch, respectively, as substrates.

Maltotriose has poor primer ability, and the malto-oligosaccharides higher than G_3 show good primer ability, for the synthetic reaction of phosphorylase[8–14]. The G_3-primed reaction is complicated because the initially formed G_4 and higher saccharides are better substrates than G_3, and it is the elongation reaction of these higher saccharides that is measured rather than the reaction of G_3. Thus, the time course of the reaction is an accelerating curve. The enzyme β-amylase also has greatly reduced activity for G_3, as compared to higher malto-oligosaccharides[20,21] so that its incorpora-

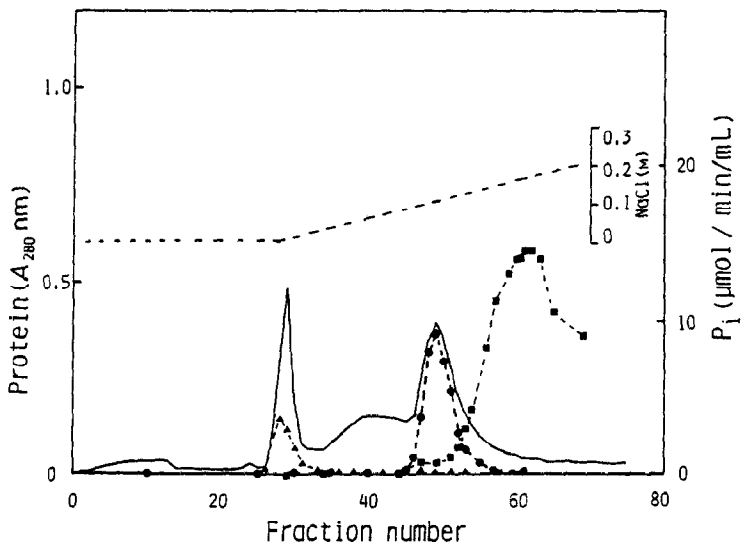

Fig. 2. Column chromatography of potato phosphorylase on DEAE-Toyopearl. Fractions of 5 mL were taken. The solid line shows protein concentration, and the dotted lines the activities of phosphorylase (–●–), phosphatase (–▲–), and D-enzyme (–■–). Phosphatase activity was determined by measuring the absorbance at 410 nm due to *p*-nitrophenol released from *p*-nitrophenyl phosphate.

tion in the digest in sufficient quantity brings about the rapid hydrolysis of the synthesized G_4 to G_2, thereby removing the former before it can be elongated to G_5. Therefore, the measurement of the released phosphate from G-1-P is exclusively a measurement of the addition of glucose to G_3.

The minimum quantity of β-amylase sufficient for the rapid degradation of G_4 was estimated as follows: Fig. 1 shows time courses of the G_3-primed reaction in the absence (concavely curved) and in the presence of β-amylase (0.4 unit/mL, slightly curved; 4 units/mL, linear). The addition of more than 4 units/mL of β-amylase caused no further change. Finally, exactly the same rate was obtained when G_3 was preincubated with 4 units/mL of β-amylase for 30 min before the synthetic reaction was initiated, indicating no significant degradation of the trisaccharide by the amylase. Thus, the presence of about 4 units/mL of β-amylase is sufficient to eliminate additional reactions with higher saccharides produced, and thus give the true time course of the reaction with G_3.

In the G_3-primed reaction the concentration of phosphorylase was tenfold higher than that in reactions employing the higher saccharides. At this enzyme concentration, no reaction was observed when the phosphorylase was incubated with G-1-P only (no primer). Preincubation of the phosphorylase and primer G_3 for 30 min, prior to the addition of G-1-P, did not affect the G_3-primed reaction at all. This indicates that the enzyme preparation was free of D-enzyme.

There have been several reports of uncommon behaviors in reactions involving carbohydrolases and poor substrates, such as maltose and maltotriose[22-26]. Among these it is pertinent to cite the reaction of *Bacillus subtilis* saccharifying α-amylase with purified maltose[24], which exhibits an accelerating time course similar to that of the G_3-primed reaction of phosphorylase. However, differing from the phosphorylase case, the steady-state rate of the reaction shows a strong sigmoidal dependency on the concentration of the substrate maltose[25]. In this instance, the phenomena are mainly attributable to transfer action of the bacterial enzyme[24,26].

Determination of kinetic parameters for G_3 and higher malto-oligosaccharides. — As mentioned above, the true rate of the G_3-primed reaction was measured from the slope of linear plots of the time course in the presence of β-amylase. The plotting of (s/v) against s then gave a straight line, and the K_m value of 9.4 mM could be determined by extrapolation of the line. We also estimated the values of both K_m and V/e, and their standard deviations by application of the Michaelis-Menten equation and a nonlinear least squares method[27]. The two methods gave the same value of K_m for G_3. With the higher saccharides, linear time courses were observed in the ordinary reactions (without β-amylase), and the initial rates were measured from the slopes of the plots. Then, the kinetic parameters were calculated by the same statistical method. The results are shown in Table I.

The phosphorolytic activity of potato phosphorylase was determined by a coupled assay, involving measurement of absorbance at 340 nm due to NADPH, essentially as described by Lee and Braun[11]. The addition of a small amount (4 μM) of glucose 1,6-diphosphate was required to raise the rate of the first coupling reaction (mutase),

TABLE I

Kinetic parameters of potato phosphorylase action on malto-oligosaccharides

Substrate	*Synthesis*		*Phosphorolysis*	
	K_m (*m*M)	V/e[a]	K_m (*m*M)	V/e[a]
G_3	9.40 ± 0.8	0.78 ± 0.03		
G_4	0.72 ± 0.11	40 ± 3	0.77 ± 0.18	0.015 ± 0.002
G_5	0.40 ± 0.04	35 ± 1	0.47 ± 0.07	14 ± 1
G_6	0.23 ± 0.02	40 ± 1	0.35 ± 0.05	17 ± 2
G_7	0.14 ± 0.02	46 ± 2	0.11 ± 0.01	18 ± 2
G_8	0.10 ± 0.01	49 ± 3	0.11 ± 0.02	21 ± 2

[a] *V/e* = μmol of released P_i/min/mg of protein.

because we used G-1-P purified on carbon columns[28]. The initial rate was estimated from the increase in the absorbance between at 5 min and 15 min after the reaction was started. The K_m values for phosphorolytic reactions with malto-oligosaccharides (G_4–G_8) were all determined by the above-mentioned statistical method (see Table I).

Phosphorolytic reactions of saccharides smaller than G_4 were too slow to detect under the conditions of the G_4 reaction. In the synthetic direction no G_2-primed reaction was observed. There are small differences between the values of K_m found for the synthetic and phosphorolytic reactions of each of the higher saccharides G_4–G_8. Large differences in values of the parameters K_m and *V/e* were seen on going from G_3 to G_4 as substrates for the synthetic reaction, and from G_4 to G_5 for the phosphorolytic reaction. With the higher saccharides it may be noted that the values of *V/e* remain fairly constant, while the values of K_m have a tendency to decrease with increasing d.p. These tendencies for higher saccharides have also been found in studies of other plant enzymes[11,12,14].

As can be seen by Eq. *1*, a synthetic reaction with G_3 is the reverse of a phosphorolytic reaction with G_4. Thus, when the number *n* is equal to or more than three, Eq. *1* is applicable. A possible explanation for the characteristic kinetics of phosphorylase action on G_3 as contrasted with higher saccharides can be based on a subsite model, as shown in Fig. 3. In the figure, the upper scheme represents the reaction system G_4 + G-1-P $\rightleftharpoons$ G_5 + P_i and the lower scheme G_3 + G-1-P $\rightleftharpoons$ G_4 + P_i. The schemes are based on the assumptions that the glucose moiety of G-1-P would bind at the common binding site (subsite 1) adjacent to the nonreducing glucose residue of the glucan cosubstrate, while the phosphate moiety would bind at a specific site different from the glucan binding site (subsites 2–*n*). This model is essentially the one proposed by French[10] in 1953, and it seems to be consistent with recent schemes for the reaction mechanism[1-3] that involves the participation of PLP.

The upper reaction as catalyzed by phosphorylase proceeds much more rapidly than the lower in both the forward and reverse directions. The two reaction systems

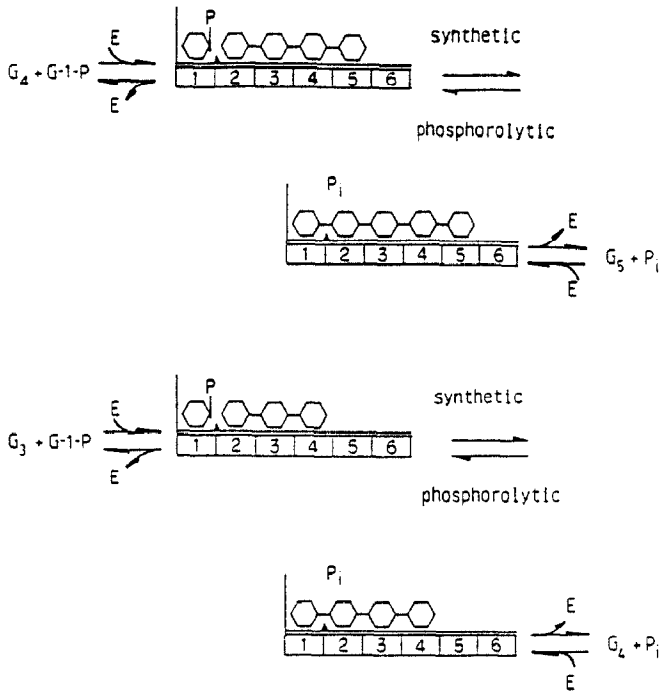

Fig. 3. Schematic model of the synthetic and phosphorolytic reactions catalyzed by glucan phosphorylase. A hexagon indicates a glucose residue of a malto-oligosaccharide; the vertical line indicates a barrier, and the wedge the catalytic site of the enzyme. The numbers in the boxes are the subsite numbers, counting from the nonreducing-end residue of the bound phosphorolysis substrate.

differ in whether or not the fifth subsite is occupied by a glucose residue of the substrate. Thus it may be concluded that the binding of the glucose residue to the fifth subsite is responsible for greatly increasing the rates of the synthetic and phosphorolytic reactions. The rate parameters for saccharides higher than G_3 in the synthetic reaction (or G_4 in the phosphorolytic reaction) did not vary strongly with increasing d.p. Thus, binding to subsites beyond the fifth, if any, would make little contribution to an increase in the rate.

This is the first determination of the kinetic parameters K_m and V/e for maltotriose in the synthetic reaction catalyzed by potato phosphorylase, made possible by incorporating a sufficient quantity of β-amylase in the digest. All the kinetic parameters for the series of malto-oligosaccharides G_4–G_8 were also determined in both the synthetic and phosphorolytic directions.

EXPERIMENTAL

Preparation of malto-oligosaccharides and potato phosphorylase. — Malto-oligosaccharides G_4 through G_7 were obtained from an acid hydrolyzate of cyclomaltoheptaose by chromatography on a cellulose column[29]. Each oligosaccharide was pure (more than 99%) by h.p.l.c. Malto-octaose was donated by Dr. T. Nakakuki of Nihon Shokuhin Kako Co., Ltd.

Purification of the potato enzyme[18] was carried out on a column (1.8 × 12 cm) of DEAE-Toyopearl. Phosphorylase was separated effectively from D-enzyme by elution with a linear gradient of 0 to 0.3M NaCl in 2 mM citrate buffer, pH 6.7 (Fig. 2). The pooled phosphorylase fractions were concentrated in a collodion bag (Sartorius GmbH) to a specific activity of 29 units/mg. On polyacrylamide gel disc electrophoresis at pH 8.0, the preparation showed a single protein band coinciding in position with a single enzyme band, detected by activity staining[30]. One unit of phosphorylase is defined as the amount of the enzyme that forms 1 μmol of inorganic phosphate per minute from 10mM G-1-P and 1% soluble starch in 50mM citrate buffer, pH 6.2, at 30°.

Protein determinations were carried out as described by Lowry *et al.*[31].

Synthetic reaction with maltotriose as substrate. — A mixture of G_3 (5–25mM), 10mM G-1-P, and 16 units/mL of sweet potato β-amylase[32] in 4 mL of 40mM citrate buffer, pH 6.2, was preincubated at 30° for 10 min. The phosphorylase reaction was started by the addition of 0.1 mL (2.1 units) of the potato enzyme, to give a final concentration of 0.5 unit/mL. An aliquot of 1 mL was taken for analysis every 10 min and added to 0.5 mL of 5% trichloroacetic acid to terminate the reaction. Inorganic phosphate released was determined by the Fiske–Subbarow method[33].

Synthetic reactions with higher malto-oligosaccharides. — The reaction was run with five or six different concentrations of each oligosaccharide, the highest level being 1–3 times K_m. A mixture of malto-oligosaccharide, 10mM G-1-P, and 0.3 unit (final 0.075 unit/mL) of phosphorylase in 4 mL of 40mM citrate buffer, pH 6.2, was incubated at 30° for 10 min. At intervals of 5 min aliquots of 1 mL were taken for analysis by the Fiske–Subbarow method.

Phosphorolytic reaction monitored by coupled enzyme assay[11,28]. — For the enzymatic analysis, phosphoglucomutase (rabbit muscle, 330 units/mg, Sigma Chemical Co.) and glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, 320 units/mg, Wako Pure Chemical Industries, Ltd.) were used. A mixture of 25 mL of 100mM NADP solution containing 0.23mM glucose 1,6-diphosphate, 25 μL of the dehydrogenase (1.9 units), 25 μL of the mutase (2.3 units), 50 μL of the phosphorylase (0.075 unit), 0.71 mL of distilled water, and 0.2 mL of 47mM NaH_2PO_4 solution containing 0.3M sodium glycerophosphate, 125mM $MgCl_2$, and 10mM EDTA·2 Na, pH 6.2, was preincubated at 30° for 5 min. The reaction was started by the addition of 0.1 ml of malto-oligosaccharide (G_5–G_8) solution. The formation of NADPH was recorded continuously at 340 nm in a Shimadzu UV-265 spectrophotometer, equipped with a cell holder maintained at 30°. In the reaction with G_4, 2.7 units of phosphorylase was used.

Assay for D-enzyme activity employing phosphorylase as a secondary enzyme. — The principle of this method has been described in the previous paper[17]. A mixture of 0.1 mL of purified G_3 (20 mM) in 75mM Tris–maleate buffer, pH 7.0, and 0.1 mL of sample to be assayed was incubated for 30 min at 30°. The mixture was heated for 3 min to stop the reaction. Then, the phosphorylase reaction was started by the addition of 10 μmol of G-1-P and 0.45 unit of potato phosphorylase in 1 mL of 40mM citrate buffer, pH 6.0. After incubation for 5 min at 30°, 5 mL of cold 0.5M H_2SO_4 was added to the reaction mixture. D-Enzyme activity could be related to the released phosphate, measured at 740 nm by the Fiske–Subbarow method.

REFERENCES

1 S. G. Withers, N. B. Madsen, B. D. Sykes, M. Takagi, S. Shimomura, and T. Fukui, *J. Biol. Chem.*, 256 (1981) 10 759–10 762.
2 M. Takagi, T. Fukui, and S. Shimomura, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 3716–3719.
3 D. Palm, H. W. Klein, R. Schinzel, M. Buehner, and E. J. M. Helmreich, *Biochemistry*, 29 (1990) 1099–1107.
4 S. Shimomura and T. Fukui, *Biochemistry*, 19 (1980) 2287–2294.
5 K. Nakano and T. Fukui, *J. Biol. Chem.*, 261 (1986) 8230–8236.
6 T. Fukui, S. Shimomura, and K. Nakano, *Mol. Cell. Biochem.*, 42 (1982) 129–144.
7 C. Weibull and A. Tiselius, *Ark. Kemi*, 19A, no. 19 (1945) 1–25.
8 J. M. Bailey, W. J. Whelan, and S. Peat, *J. Chem. Soc.*, (1950) 3692–3694.
9 W. J. Whelan and J. M. Bailey, *Biochem. J.*, 58 (1954) 560–569.
10 D. French and G. M. Wild, *J. Am. Chem. Soc.*, 75 (1953) 4490–4492.
11 E. Y. C. Lee and J. J. Braun, *Arch. Biochem. Biophys.*, 156 (1973) 276–286.
12 M. Steup and C. Schächtele, *Planta*, 153 (1981) 351–361.
13 T. Suganuma, K. Beppu, S. Fujimoto, and T. Nagahama, *Agric. Biol. Chem.*, 47 (1983) 2961–2963.
14 Y. Nakamura and M. Imamura, *Phytochemistry*, 22 (1983) 2395–2399.
15 W. J. Whelan, J. M. Bailey, and P. J. P. Roberts, *J. Chem. Soc.*, (1952) 1293–1298.
16 S. Peat, W. J. Whelan, and W. R. Rees, *Nature*, 172 (1953) 158.
17 T. Suganuma, S. Setoguchi, S. Fujimoto, and T. Nagahama, *Carbohydr. Res.*, 212 (1991) 201–212.
18 A. Kamogawa, T. Fukui, and Z. Nikuni, *J. Biochem. (Tokyo)*, 63 (1963) 361–368.
19 I. Maeda, in The Amylase Research Society of Japan (Ed.), *Handbook of Amylases and Related Enzymes*, Pergamon Press, Oxford, 1988, pp. 164–169.
20 J. A. Thoma and D. E. Koshland, Jr., *J. Biol. Chem.*, 235 (1960) 2511–2517.
21 T. Suganuma, M. Ohnishi, K. Hiromi, and Y. Morita, *Agric. Biol. Chem.*, 44 (1980) 1111–1117.
22 T. Suganuma, M. Ohnishi, R. Matsuno, and K. Hiromi, *J. Biochem. (Tokyo)*, 80 (1976) 645–648.
23 J. F. Robyt and D. French, *J. Biol. Chem.*, 245 (1970) 3917–3927.
24 H. Fujimori, M. Ohnishi, M. Sakoda, R. Matsuno, and K. Hiromi, *J. Biochem. (Tokyo)*, 82 (1977) 417–427.
25 T. Shibaoka, T. Inatani, K. Hiromi, and T. Watanabe, *J. Biochem. (Tokyo)*, 77 (1975) 965–968.
26 T. Suganuma, Doctoral Thesis (with Prof. K. Hiromi), Kyoto University, 1978.
27 W. W. Cleland, in D. L. Purich (Ed.), *Methods in Enzymology*, Vol. 63, Academic Press, New York, 1979, pp. 103–138.
28 H. U. Bergmeyer and G. Michal, in H. U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Vol. 3, Verlag Chemie, Weinheim, 1974, pp. 1233–1237.
29 J. A. Thoma, H. B. Wright, and D. French, *Arch. Biochem. Biophys.*, 85 (1959) 452–460.
30 C. H. Davis, L. H. Schliselfeld, D. P. Wolf, C. A. Leavitt, and E. G. Krebs, *J. Biol. Chem.*, 242 (1967) 4824–4833.
31 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
32 Y. Takeda and S. Hizukuri, *Biochim. Biophys. Acta*, 185 (1969) 469–471.
33 C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66 (1925) 375–400.