On the Hysteretic Response of Rabbit Skeletal Muscle Phosphorylase Kinase†

Giho Kim‡ and Donald J. Graves*,§

ABSTRACT: Progress curves of the reaction catalyzed by nonactivated phosphorylase kinase are not linear, and enzymic activities are not proportional to its concentration. With nonactivated enzyme, a fourfold increase in activity is obtained by preincubation with phosphorylase *b* and Mg^{2+}. Trypsin-activated phosphorylase kinase also shows an initial lag in its reaction. From this lag period, an initial velocity is calculated. With trypsin-activated phosphorylase kinase at 5°, an increase in the specific activity occurs with enzyme dilution and levels off at concentrations lower than 13 μg/ml. At high enzyme concentration (28 μg/ml), the Arrhenius plot shows a discontinuity with activation energies of 18,200 and 2600 cal, whereas the Arrhenius plot is linear at low enzyme concentration (7 μg/ml). The initial velocity also is affected by preincubation with phosphorylase *b*. The progress curve of the enzymic reaction becomes linear by preincubation with phosphorylase *b*, having a half-life of 2.5 min under the conditions employed. The data suggest that phosphorylase kinase is a hysteretic enzyme of which activities are controlled by a combination of substrate binding and association–dissociation of the enzyme.

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.138) is present in skeletal muscle as nonactivated (Krebs *et al.*, 1964) and activated phosphorylated forms (DeLange *et al.*, 1968). The proportion of these forms present in muscle before and after stimulation (neural, pharmacological, or hormonal) is usually assessed by measurement of enzyme activity at different pH values. Nonactivated phosphorylase kinase is characterized by its low ratio of activities (pH 6.8:8.2 < 0.05) and the activated phosphorylated form by its higher ratio of activities (pH 6.8:8.2 ~ 0.5).

In the study of the nonactivated form of phosphorylase kinase, a lag is seen in the catalyzed reaction at pH 6.8 (Krebs *et al.*, 1964). In this manuscript, we examine the lag in the reaction using nonactivated and tryptic activated phosphorylase kinase (Krebs *et al.*, 1964). The time lag of product formation in response of an enzyme to a rapid change in concentrations of substances, *e.g.*, substrates, products, modifiers, enzyme, is defined as hysteresis (Frieden, 1970). A mechanism for the hysteresis is proposed, and its significance is discussed in relation to the measurement of enzyme activity at different pH values.

Experimental Procedures

Materials. Crystalline skeletal muscle phosphorylase *b* was prepared by the method described by Fischer and Krebs (1958), but mercaptoethanol was substituted wherever cysteine was required. The concentration of phosphorylase *b* was determined spectrophotometrically at 280 nm using an absorbancy index (1% × cm^{-1}) of 13.2 (Kastenschmidt *et al.*, 1968). Concentration was expressed as molarity using a molecular weight of 100,000 for a monomer (Cohen *et al.*, 1971). Nonactivated phosphorylase kinase was prepared by the modified procedure described by Brostrom *et al.* (1971). Unless otherwise indicated, the phosphorylase kinase used in our studies was the peak II fraction from Sepharose 4B chromatography. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (1964) with minor modifications (Walsh *et al.*, 1971). Trypsin-activated phosphorylase kinase (Krebs *et al.*, 1964) was prepared by incubating nonactivated phosphorylase kinase (1–2 mg/ml) with trypsin for 10 min at pH 6.8 and 30° at weight ratio of the enzyme to trypsin of 500 to 1. A sixfold excess of soybean trypsin inhibitor on a weight-to-weight basis was added to stop the reaction. All other reagents employed were analytical grade available commercially.

Methods. Phosphorylase activities were determined by the method of Cori *et al.* (1943). Phosphorylase kinase activities were determined by the method described previously (Krebs, 1966) with a slight modification in that 0.015 M cysteine was replaced by 0.04 M glycerol-P–0.03 M 2-mercaptoethanol at pH 6.8. Approximately 30 sec after making a dilution of phosphorylase kinase at the assay temperature, the reaction was started by adding the enzyme. The concentration of phosphorylase *b*, MgATP, and buffer was used as specified. After stopping the reaction by dilution in cold 0.04 M glycerol-P–0.03 M 2-mercaptoethanol buffer at pH 6.8, phosphorylase *a* activity was measured. Phosphorylase kinase units were calculated according to Krebs (1966). The amount of ^{32}P bound to phosphorylase in the phosphorylase kinase reaction was determined by the filter paper method of Reimann *et al.* (1971) and was used as an assay for the early experiments.

Results

To do meaningful steady-state kinetics with an enzyme, it is necessary to show that initial velocity can be measured and that enzymic activity is proportional to enzyme concentration. Figure 1 shows some progress curves obtained with nonactivated phosphorylase kinase at different enzyme concentra-

† From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. *Received December 11, 1972.* Supported by Research Grant GM-09587 from the National Institutes of Health, U. S. Public Health Service. Journal Paper J-7469 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 1843.

‡ Present address: Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, N. Y. 11203.

§ Recipient of a Research Career Development award of the U. S. Public Health Service (Grant GM6753).

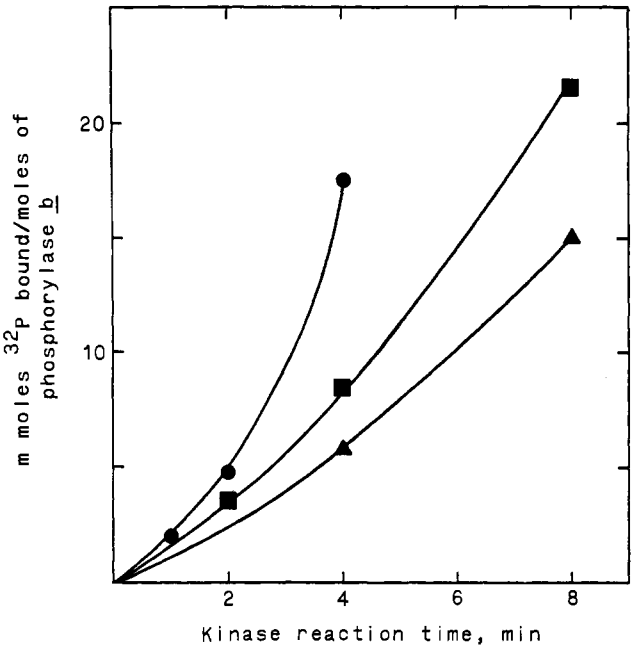

FIGURE 1: Effect of enzyme concentration on nonactivated phosphorylase kinase activity. Reaction was initiated by the addition of 20 μl of nonactivated phosphorylase kinase in 0.02 M Hepes–0.02 M 2-mercaptoethanol buffer at pH 7.7 to reaction mixture containing 20 μl of phosphorylase *b* (3.4×10^{-4} M in the above buffer), 20 μl of Mg–[^{32}P]ATP (4.5–0.75 mM) and 10 μl of buffer at 30°; 20 μl of the reaction mixture was withdrawn at the indicated times and ^{32}P activity incorporated to phosphorylase was determined. (●) 2 μg/ml of enzyme; (■) 1 μg/ml of enzyme; (▲) 0.5 μg/ml of enzyme.

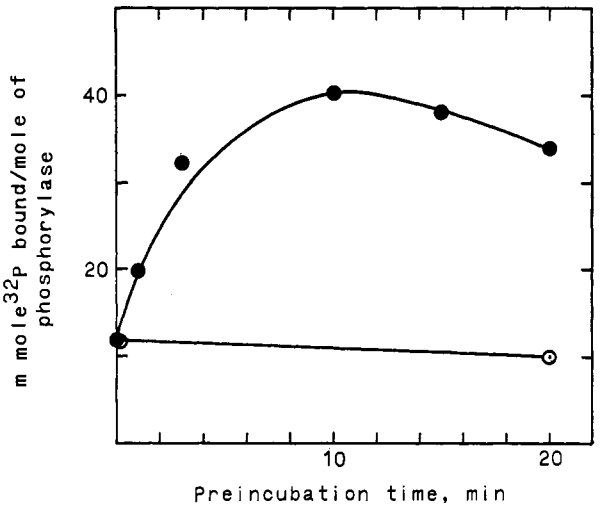

FIGURE 2: Effect of preincubation with phosphorylase *b* and Mg^{2+} ions on nonactivated phosphorylase kinase activity; 20 μl of nonactivated phosphorylase kinase, 7 μg/ml in 0.02 M Hepes–0.02 M 2-mercaptoethanol buffer at pH 7.7, was preincubated with 20 μl of phosphorylase *b* (3.4×10^{-4} M in the above buffer), 10 μl of $Mg(Ac)_2$ (9 mM), and 10 μl of the buffer at 30°. The reaction was intiated by adding 10 μl of 1.5 mM [^{32}P]ATP at the times indicated. After 2 min of reaction at 30°, 50 μl was removed for counting ^{32}P activity incorporated into phosphorylase. (●) Preincubation with phosphorylase *b* and Mg^{2+}; (○) preincubation in buffer.

tions in Hepes[1] buffer. The lines are curvilinear, and the velocities are not proportional to enzyme concentration. The data show that, as the enzyme concentration is decreased and the assay time is increased, proportionally more product is formed than expected; *e.g.*, more product is released at 1 μg/ml of enzyme (squares) in 4 min than that from enzyme at 2 μg/ml (circles) in 2 min.

The phosphorylase kinase reaction was started by the addition of enzyme (the usual method), phosphorylase *b*, or ATP to determine whether the order of addition could affect the shape of the progress curves. Higher activities were seen when ATP or phosphorylase *b* was added last. Figure 2 shows the extent of activation obtained after different times of incubation of phosphorylase kinase with phosphorylase *b* and Mg^{2+}. In the upper curve, the reaction was started at the indicated time with ATP. The lower curve represents enzyme incubated in buffer alone and shows that no inactivation or activation occurs over this period. Also, preincubation with Mg^{2+} produces no activation. Enzyme preincubated with phosphorylase *b* and Mg^{2+} showed essentially a fourfold increase in activity and seemed to decline slightly in activity after 10 min. Preincubation with phosphorylase *b* alone also activates phosphorylase kinase, but a slightly higher activation was obtained in the presence of phosphorylase *b* and Mg^{2+}. The effect of preincubation of phosphorylase kinase for 15 min on a progress curve is presented in Figure 3. The activation is apparent, and some change occurs in the shape of the progress

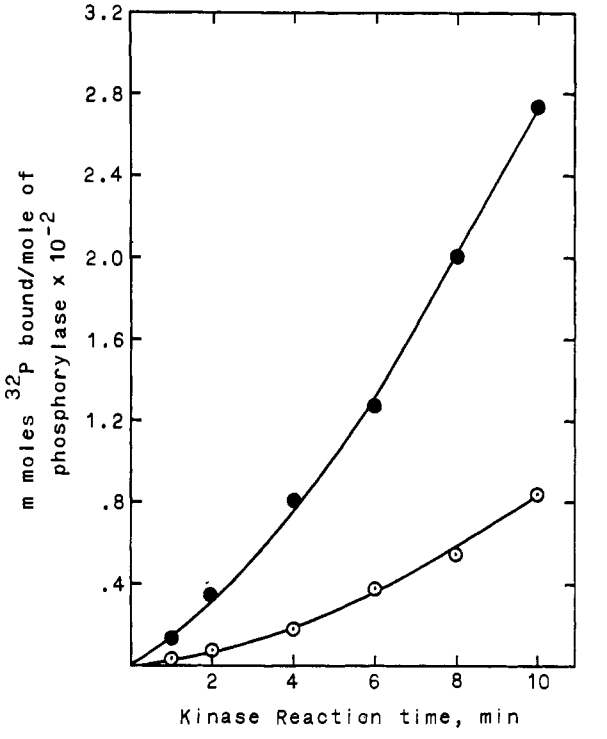

FIGURE 3: Time course of nonactivated phosphorylase kinase preincubated with phosphorylase *b* and Mg^{2+}. The reaction mixture contained 100 μl of nonactivated phosphorylase kinase, 7 μg/ml in 0.02 M Hepes–0.02 M 2-mercaptoethanol at pH 7.7, 100 μl of phosphorylase *b* (3.4×10^{-4} M in the above buffer), 50 μl of $Mg(Ac)_2$ (4.5 mM), 50 μl of the buffer, and was preincubated for 15 min at 30°. The reaction was initiated by adding 50 μl of [^{32}P]ATP (1.5 mM) and 50 μl of the reaction mixture was removed at the times indicated for counting ^{32}P activity incorporated into phosphorylase. (●) Preincubated with phsophorylase *b* and Mg^{2+}; (○) no preincubation.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; cAMP, cyclic adenosine 3′,5′-monophosphate.

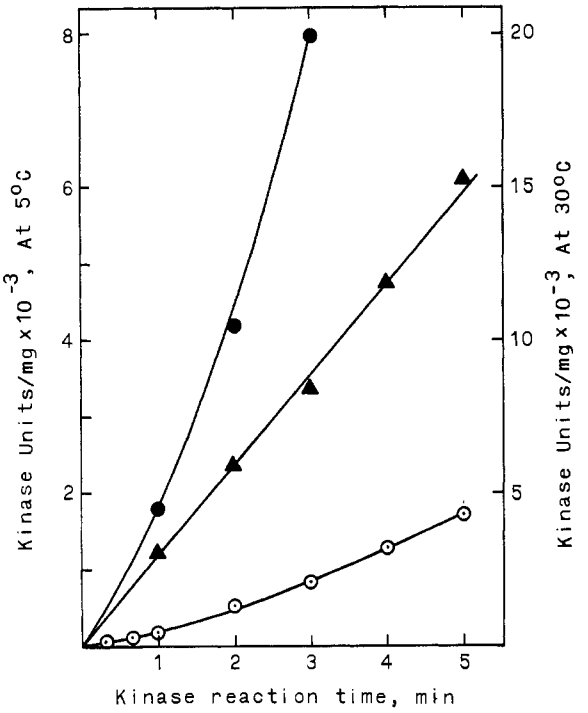

FIGURE 4: Time course of the trypsin-activated phosphorylase kinase reaction at 30 and 5°. The reaction mixture was as described by Krebs (1966). Activity at pH 6.8 at 30° with 113 μg/ml of enzyme (●); activity at pH 6.8 at 5° with 34 μg/ml of enzyme (○); activity at pH 8.2 at 5° with 34 μg/ml of enzyme (▲).

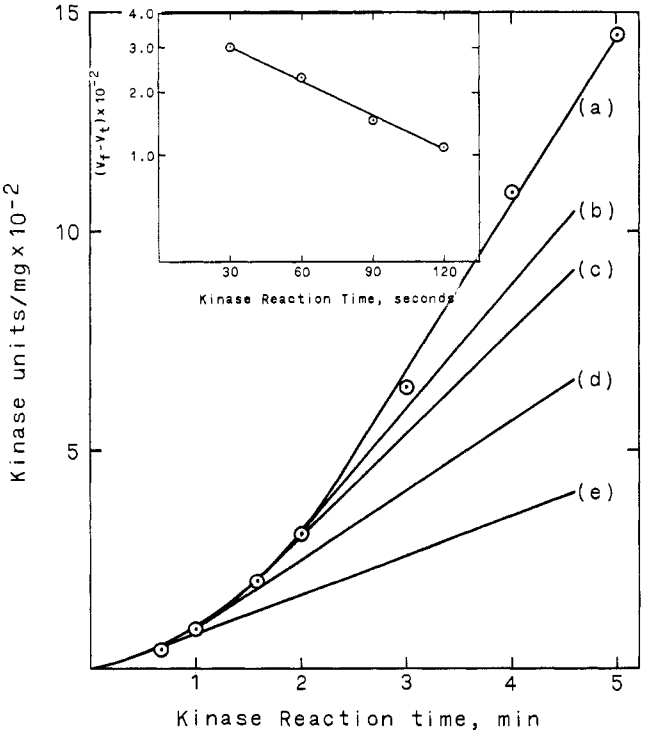

FIGURE 5: Estimation of initial velocity of the trypsin-activated phosphorylase kinase reaction. Time course of the reaction at pH 6.8 and 5°. The reaction mixture was as described by Krebs (1966). Final velocity, V_f, and velocities at time t, V_t, for the kinase reaction were estimated from the slopes of the curve at different times: (a) for V_f; (b), (c), (d), and (e) for V_t at 120, 90, 60, and 30 sec, respectively. Plot of log $(V_f - V_t)$ *vs.* reaction time.

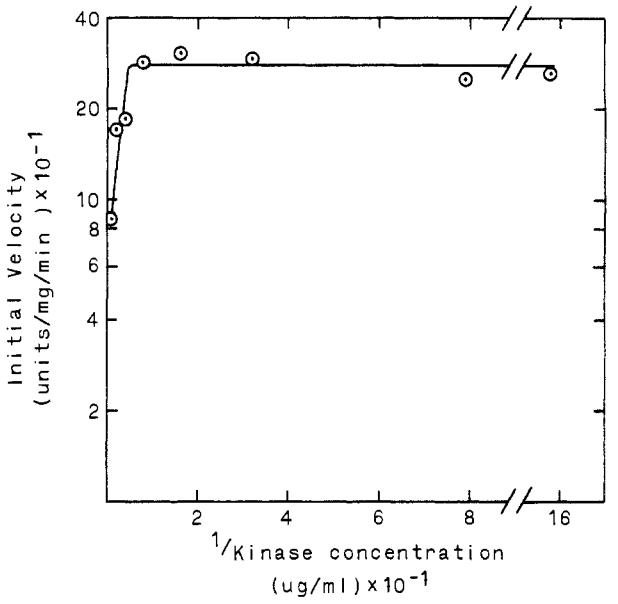

FIGURE 6: Effect of the concentration of trypsin-activated phosphorylase kinase on initial velocity. The reaction mixture at 5° was as described (Krebs, 1966) at pH 6.8 except the phosphorylase *b* was reduced from 4 × 10⁴ to 1 × 10⁴ units per ml. From a set of progress curves obtained, initial velocities corresponding to each kinase concentration were estimated by using the equation given in the text. Then initial velocities were plotted against the reciprocal of enzyme concentration.

curve upon preincubation with phosphorylase *b* and Mg^{2+}, but the nonlinearity is still present.

The reason for the nonlinear progress curve has not been understood. Since phosphorylase kinase can phosphorylate itself and purified nonactivated phosphorylase kinase contains some protein kinase, it is possible that the increase in activity or the initial lag is related to the conversion of inactive kinase to its activated phosphorylated form in the assay. To gain some information about this, studies were undertaken with trypsin treated phosphorylase kinase because it is already activated (Krebs *et al.*, 1964), and we found that no further activation took place by incubating this form of the enzyme with either cAMP alone or cAMP and protein kinase. Therefore, if the lag in the reaction is due to activation by phosphorylation, no lag should be seen in the reaction with trypsin-activated kinase. Figure 4 shows the progress curves at two different temperatures. Surprisingly, the progress curves were not linear at pH 6.8, and this nonlinearity became more pronounced at the lower temperature. At pH 8.2, progress curves of the reaction were almost linear even at the low-temperature assay illustrated in the figure. Also, the ratio of activities at pH 6.8 : 8.2 at 5° was low. These unexpected results prompted us to focus our studies on the initial velocity of the reaction in order to gain more insight on the lag of the phosphorylase kinase catalyzed reaction. Figure 5 illustrates the way in which the velocities were determined. From the plot of the progress curve obtained from the enzyme reaction, tangents were constructed to the points at which product was measured. From these slopes, V_t, and the final slope, V_f, and utilizing the equation developed by Frieden (1970) for a hysteretic response, $V_t = V_f + (V_0 - V_f)e^{-kt}$, the rate constant, k, was calculated. The insert shows that the results can be described by this equation. Thus, from these data, the initial velocity, V_0, can be obtained.

Studies were continued using trypsin-activated kinase be-

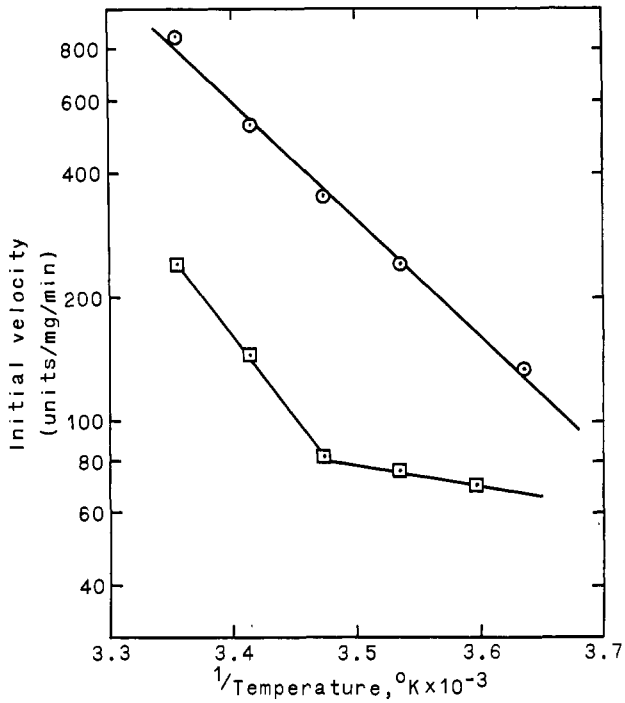

FIGURE 7: Effect of temperature on the initial velocity of trypsin-activated phosphorylase kinase at pH 6.8. Reactions were at 5° as in Figure 4. (○) 7 μg/ml; (□) 28 μg/ml. From a set of progress curves obtained initial velocities were estimated by using the equation given in the text. Then the log of initial velocity was plotted against the reciprocal of the temperature of the reaction.

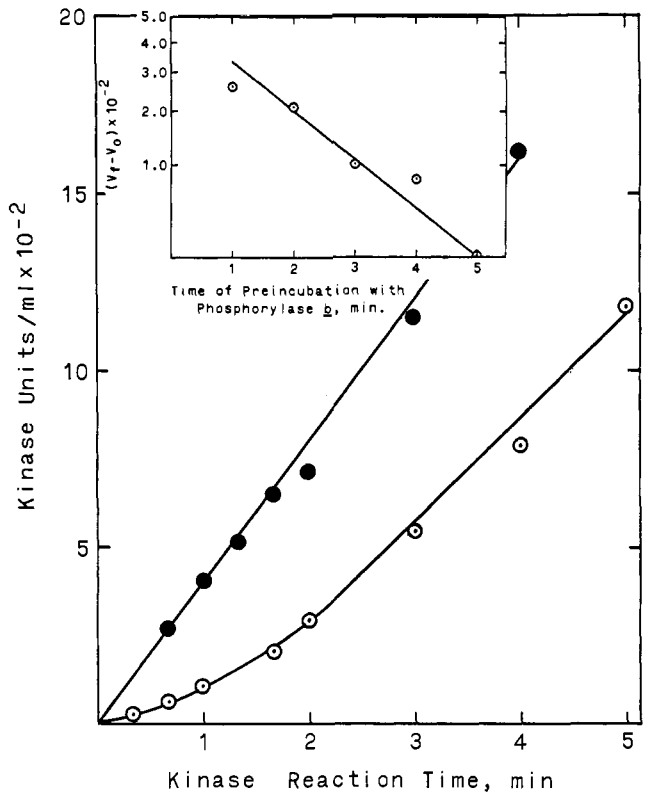

FIGURE 8: Effect of phosphorylase *b* on the velocity of trypsin-activated phosphorylase kinase at/pH 6.8 and 5°. Reaction mixture containing 0.2 ml of 0.125 M Tris–0.125 M glycerol-P at pH 6.8, 0.2 ml of phosphorylase *b* (1×10^4 units/ml), and 0.1 ml of tryspin-activated phosphorylase kinase (248 μg/ml) was preincubated for 0, 1, 2, 3, and 5 min at 5°, the reaction was initiated by adding 0.1 ml of MgATP (60–18 mM). Progress curve of enzyme reaction at pH 6.8 and 5°. (●) 5-min preincubation with phosphorylase *b;* (○) no preincubation. From a set of progress curves at different preincubation times, initial velocities were estimated by using the equation in the text. Plot of log $(V_f - V_0)$ *vs.* time of preincubation.

cause it has been established that this enzyme can dissociate to lower molecular weight forms at low enzyme concentration (Graves *et al.*, 1973). If one of the factors responsible for hysteresis is an association–dissociation process (Frieden, 1970), then one would expect that the lag of the reaction would depend upon enzyme concentration. On the other hand, if hysteresis is due simply to a change in the conformation of the enzyme, no dependence of initial velocity should be seen at different enzyme concentrations. Figure 6 shows the effect on initial velocities of trypsin-activated enzyme with varying enzyme concentration, 130 μg to 0.6 μg/ml of reaction mixture at pH 6.8 and 5°. The data show that no change in the specific activities of the enzyme occurs when the enzyme concentration is 13 μg or less and that specific activities decrease sharply when the concentration is increased beyond 25 μg. These data then suggest that a dissociation of the enzyme is responsible for the lag of the reaction catalyzed by the enzyme.

To further substantiate the findings that specific activity is dependent upon enzyme concentration, the effect of temperature on the reaction was studied. It was reasoned that an Arrhenius plot would deviate from linearity under conditions where different enzyme forms were present. From the results of Figure 6, this effect should be pronounced at high concentrations where specific activity depends upon enzyme levels. Figure 7 shows temperature effects on specific activities with enzyme concentrations, of 28 μg and 7 μg/ml. The data show that the change in specific activity at the lower enzyme concentration is linear (activation energy equals 9900 cal), whereas a marked discontinuity occurs in the plot at about 15° with the higher enzyme concentration (with activation energies of 18,200 and 2600 cal for the upper and lower limb, respectively).

The effect of phosphorylase *b* on the hysteretic response of trypsin-activated phosphorylase kinase was tested. Figure 8 shows that preincubation with phosphorylase *b* gives higher enzymic activity, and the results are similar to those obtained with nonactivated phosphorylase kinase (Figure 3). In this case, in addition to activation the progress curves become linear after 5-min preincubation. To determine the half-life for this process, phosphorylase kinase was preincubated with phosphorylase *b* for different time intervals before initiating the reaction with MgATP. The insert shows that the increase of initial velocity follows first-order kinetics with respect to preincubation time and that the half-life for the progress curve to become linear is 2.5 min under the experimental condition. The effect of preincubation could be explained if it is assumed that an equilibrium exists between enzyme forms and that the activated state has the highest affinity for phosphorylase *b*. Activation of glycogen phosphorylase [*e.g.*, preincubation with glycogen (Wang *et al.*, 1965)] has been explained by a difference in the affinities of tetrameric and dimeric forms for glycogen (Huang and Graves, 1970). If the lag then, in the phosphorylase kinase reaction is due to the conversion of enzyme to a form of higher affinity, this lag should depend on phosphorylase *b* concentration. In Figure 9, progress curves are shown with 4×10^{-4} and 0.5×10^{-4} M phosphorylase *b*. The data clearly show the lag is less pronounced at the higher substrate concentration indicating that such a mechanism does exist.

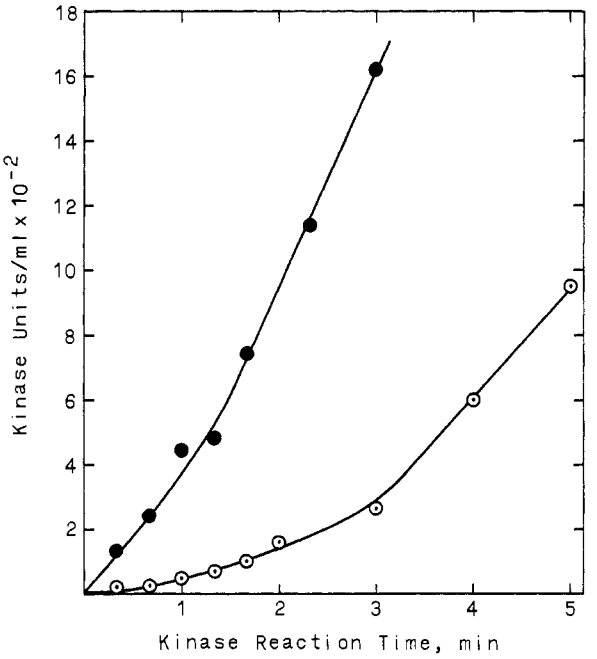

FIGURE 9: Effect of phosphorylase *b* concentration on the log of the trypsin-activated phosphorylase kinase reaction. To 0.5 ml of reaction mixture at 5° containing 0.1 ml of 0.125 M Tris–0.125 M glycerol-P at pH 6.8, 0.3 ml of phosphorylase *b*, and 0.1 ml of MgATP (60–18 mM), 0.1 ml of trypsin-activated phosphorylase kinase (248 μg/ml) was added to initiate the reaction. Progress curves of the reaction with 4×10^{-4} M phosphorylase *b* (●); 5×10^{-5} M phosphorylase *b* (○).

In Table I, activities of phosphorylase kinase under various assay conditions are shown. To see whether the parameters that affected initial velocity of trypsin-activated phosphorylase kinase also have an effect on the activity ratio at pH 6.8–8.2, activities of trypsin-activated enzyme were measured at two different enzyme concentrations and temperatures. The results show that at 10° and pH 6.8 the specific activity at low enzyme concentration (0.18 μg/ml) is about four times higher than that at higher enzyme concentration (45 μg/ml) and that the specific activities at pH 8.2 are the same regardless of enzyme concentration. Thus, the differences in specific activities at pH 6.8 but not at pH 8.2 resulted in activity ratios (pH 6.8 : 8.2) of 0.4 and 0.14 for the lower and higher enzyme concentrations, respectively. At the normal assay temperature, 30°, the ratio of activities is higher as anticipated (Krebs *et al.*, 1964). During phosphorylase kinase preparation, the first peak eluted from the Sepharose 4B column, which is considered a polymeric form of nonactivated phosphorylase kinase (Hayakawa *et al.*, 1973), usually is discarded because of its low specific activity. As shown in Table I, by trypsin attack the specific activity of the first peak can be increased by 40- and 10-fold at pH 6.8 and 8.2, respectively, whereas tryptic attack on the second peak results in 13- and 1.5-fold increases in its catalytic activities at pH 6.8 and 8.2, respectively. The data clearly show that, under the various assay conditions tested, activities at pH 6.8 were affected to a greater extent than those at pH 8.2.

TABLE I: Effect of Various Assay Conditions on Phosphorylase Kinase Activity.

	Assay Conditions[a]			
Enzyme Form	Enzyme Concn (μg/ml)	pH	Act. (Units/mg), $\times 10^{-2}$	6.8:8.2
Trypsin activated	0.18	6.8	433	
	0.18	8.2	593	0.73
	0.18	6.8	40[b]	
	0.18	8.2	100[b]	0.40
	45	6.8	13.8[b]	
	45	8.2	99[b]	0.14
Peak I				
Nonactivated	2.2	6.8	3.2	
	0.22	8.2	21.9	0.15
Trypsin activated	0.22	6.8	144	
	0.22	8.2	188	0.76
Peak II				
Nonactivated	1.4	6.8	23.5	
	0.14	8.2	304	0.07
Trypsin activated	0.14	6.8	350	
	0.14	8.2	447	0.78

[a] Phosphorylase kinase reaction was initiated by adding 10 μl of enzyme in 0.04 M glycerol-P–0.03 M 2-mercaptoethanol buffer at pH 6.8 to 50 μl of the standard reaction mixture containing 20 μl of 0.125 M Tris–0.125 M glycerol-P at pH 6.8 or 8.6, 10 μl of MgATP (10–3 mM) at pH 7 and 20 μl of phosphorylase *b* (40,000 units/ml of 0.04 M glycerol-P–0.03 M 2-mercaptoethanol buffer at pH 6.8). The reaction was carried out for 5 min at 30° and stopped by making 26-fold dilutions with cold 0.04 M glycerol-P–0.03 M 2-mercaptoethanol buffer at pH 6.8. Phosphorylase *a* assay was done as described in Methods section. [b] Assay conditions are the same as in a except that initial velocity was determined from time course of the enzymic reactions at 10°.

Discussion

The fact that the reaction catalyzed by phosphorylase kinase shows hysteresis is important for a number of reasons. First, it is customary to determine *in vitro* the enzymic activity at two different pH's, 6.8 and 8.2, and from the ratio of activities, different molecular forms of phosphorylase kinase are distinguished. These ratios depend upon the source of phosphorylase kinase (Krebs *et al.*, 1964; Drummond *et al.*, 1965; Drummond and Bullward, 1970; Khono *et al.*, 1972) and also upon the buffer used in the assay; *e.g.*, nonactivated phosphorylase kinase from rabbit skeletal muscle has a ratio of activities at pH 6.8 : 8.2 of 0.04 in glycerophosphate-Tris but a ratio of 0.22 in Hepes buffer (unpublished results). In this work we have established that hysteresis is more pronounced at pH 6.8 than 8.2 for trypsin-activated phosphorylase kinase, and it depends upon temperature, phosphorylase *b* concentration, and enzyme concentration. Because of these effects and the results obtained with nonactivated phosphorylase kinase, we suggest that considerable caution should be exercised by investigators who determine the ratio of activities by product released using a single time point. Thus, to determine whether phosphorylase kinase becomes activated in response to a particular physiological stimulus, for example hormonal administration, one should use a progress curve. A second important feature of the hysteretic response is that it is dependent upon enzyme concentration. At low enzyme concentration, the specific activity of trypsin-activated phos-

phorylase kinase is increased. It has been shown that this form of phosphorylase kinase can be dissociated by a decrease in enzyme concentration at low temperatures from the 23S form to species having sedimentation constants of 9 and 6 S (Graves *et al.*, 1973). Since these forms showed enzyme activity, we interpret the effect of enzyme concentration on hysteresis to mean that an increase in specific activity with enzyme dilution is associated with enzyme dissociation. Conditions for the physical studies (Graves *et al.*, 1973) were somewhat different than that used in this work; therefore, additional physical and catalytic measurements are needed to test this hypothesis. In support of the view that high molecular weight forms are less active, we also found that polymeric phosphorylase kinase (Table I) can be activated more greatly by trypsin than the 23S form of phosphorylase kinase.

Hysteresis also has been described for the nonactivated form of phosphorylase kinase. Some similarity exists with results from trypsin-activated phosphorylase kinase (*e.g.*, activation by preincubation with phosphorylase *b*) and the effect of enzyme concentration on enzymic activity. It has not been established in this situation whether nonactivated phosphorylase kinase can dissociate at low enzyme concentrations. Nonactivated phosphorylase kinase as isolated has a high molecular weight (1.3×10^6) (Hayakawa *et al.*, 1973) and represents 1% of the soluble protein in skeletal muscle (DeLange *et al.*, 1968). On a weight basis its ratio to its substrate, phosphorylase *b*, is 1 : 2, but the reason for the high amounts of phosphorylase kinase is not known (DeLange *et al.*, 1968). As a working hypothesis, in view of the results obtained with trypsin-activated phosphorylase kinase, it might be assumed that regulation is accomplished by a self-assembly of the enzyme at high enzyme concentration to produce an inactive or less active high molecular weight form of the enzyme. After phosphorylation by protein kinase or perhaps by contact with its substrate, phosphorylase *b*, enzyme dissociation and activation could take place.

Acknowledgments

This work was initiated in the laboratory of Edwin G. Krebs while D. J. Graves was on sabbatical leave, and this author gratefully acknowledges his interest and advice in the early phases of the work. The authors are also indebted to Dr. J. A. Thomas for a generous gift of protein kinase and to Mr. George Tessmer for his technical assistance.

References

Brostrom, C. O., Hunkeler, F. L., and Krebs, E. G. (1971), *J. Biol. Chem. 246*, 1961.

Cohen, P., Duewer, T., and Fischer, E. H. (1971), *Biochemistry 10*, 2683.

Cori, C. F., Cori, G. T., and Green, A. A. (1943), *J. Biol. Chem. 151*, 39.

DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968), *J. Biol. Chem. 243*, 2200.

Drummond, G. I., Duncan, L., and Friesen, A. J. P. (1965), *J. Biol. Chem. 240*, 2778.

Drummond, G. I., and Bullward, G. (1970), *J. Neurochem. 17*, 475.

Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.

Frieden, C. (1970), *J. Biol. Chem. 245*, 5788.

Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.

Graves, D. J., Hayakawa, T., Horvitz, R. A., Beckman, E., and Krebs, E. G. (1973), *Biochemistry* (in press).

Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G. (1973), *Biochemistry* (in press).

Huang, C. Y., and Graves, D. J. (1970), *Biochemistry 9*, 660.

Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E. (1968), *Biochemistry 7*, 3590.

Khono, J. C., Jarett, L., Mayer, S. E., and Steinberg, D. (1972), *J. Biol. Chem. 247*, 4812.

Krebs, E. G. (1966), *Methods Enzymol. 8*, 543.

Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964), *Biochemistry 3*, 1022.

Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971), *J. Biol. Chem. 246*, 1986.

Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S., and Krebs, E. G. (1971), *J. Biol. Chem. 246*, 1968.

Wang, J. H., Shonka, M. L., and Graves, D. J. (1965), *Biochemistry 4*, 2296.