KINETICS OF THE ACTIVATION OF SKELETAL MUSCLE PHOSPHORYLASE a BY AMP AND INORGANIC PHOSPHATE

Fermin Sagardia

Department of Physiology and Biochemistry, Rutgers - The State University
New Brunswick, New Jersey

Received August 27, 1964

Although the activation of phosphorylase by AMP was discovered more than 20 years ago by Cori and Cori (1936,37), the mechanism of action of AMP has remained largely unexplored until recently (Helmreich and Cori (1964), Lowry, et. al. (1964), Madsen (1964)). While studying the kinetics of the activation of phosphorylase a, we have found that in the absence of AMP the reaction does not follow Michaelis-Menten kinetics when inorganic phosphate (Pi) is the variable substrate. In the presence of AMP, however, it does. We report in this paper some preliminary results concerning this phenomenon.

MATERIALS AND METHODS

Phosphorylase a was assayed by measuring the rate of formation of NADPH at 340 mμ in a coupled assay system using glucose-6-phosphate dehydrogenase (G6PDH) and phosphoglucomutase (PG1 Mu). The reaction was followed for 15 minutes at 29-30°C in a Beckman DU Spectrophotometer with an attached linear-log varicord recorder, (Model 43, Photovolt Corp.). The reaction mixture usually contained .01M Tris, 0.01 M Cysteine HCl, 2.4×10^{-4}M NADP, .01M $MgCl_2$, 1.0 mg/ml glycogen, 0.1 unit each of G6PDH and PG1 Mu, .01M Pi; total volume 3.0 ml, pH 7.5. AMP, when present, was 2×10^{-5}M. The amount of phosphorylase was variable, ranging from 1.6 to 5.0 μg. The rates measured were kept below .015 μmoles NADPH/min, in order not to exceed the capacity of the assay system. Under these conditions initial velocities were maintained throughout the reaction period, the greatest decrease in substrate concentration

from the initial value being less than 3%.

Rabbit skeletal muscle phosphorylase a, 2 x crystallized, was purchased from Worthington Biochemical Corp. Some results were repeated on 3 lots, and found in close agreement. G6PDH and PG1 Mu were obtained from Sigma Chemical Company or from Calbiochem. NADP and AMP were Sigma products.

Glycogen was obtained from Fisher Scientific Company. Since AMP was a contaminant, it was purified by treating a 5% solution twice with activated charcoal in the cold, followed by centrifugation and filtration. The concentration of glycogen was determined from an empirical curve based on light transmission at 340 mμ. The effectiveness of the purification procedure was ascertained by measuring the absorbance at 259 mμ after hydrolyzing the glycogen.

RESULTS

Figure 1 shows the results of an experiment in which the concentration of inorganic phosphate was varied and the velocity measured as described above. In the presence of 2×10^{-5}M AMP the plot of v vs. (Pi) is hyperbolic. In its absence, the curve becomes sigmoid. This is especially apparent at concentrations below 2×10^{-3}M.

Figure 2 shows a re-plot of the data from Fig. 1, using the Lineweaver-Burk plot. In the presence of AMP, the Km for Pi is $1.5\text{-}2.0 \times 10^{-3}$M. No Km can be determined in the absence of AMP, but the concentration needed for 1/2 Vmax is about 4×10^{-3}M.

As shown in Fig. 3, the data in the absence of AMP fits a particular form of the Hill equation which has been proposed by Monod, et al. (1963). In a plot of log v/Vmax-v vs. log (S), the slope gives the value of n in the equation:

$$v = \frac{V_{max}\,(S)^n}{K + (S)^n} \qquad (1)$$

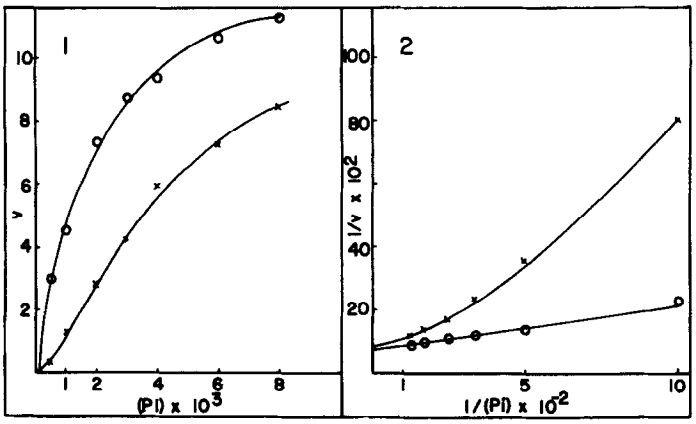

Figure 1. Velocity of reaction (in μmoles NADPH/min/μg of phosphorylase x 1000) vs. concentration of inorganic phosphate. X - X, no AMP. 0 - 0, 2 x 10^{-5}M AMP.

Figure 2. Double-reciprocal plot of the data from Fig. 1.

In several experiments the value of n was fairly constant, ranging from 1.51 to 1.65. This would suggest, among other possibilities, that there are two interacting phosphate sites. To test this assumption, the effects of arsenate were studied.

Arsenate, as in other systems, is a competitive inhibitor of phosphate. The effect of adding arsenate in the absence of AMP, however, is more complex. Figure 4 shows that in the presence of .01M arsenate, the kinetics of the reaction with respect to phosphate are normalized with a Km for phosphate of 1 x 10^{-2}M. This is a specific effect of arsenate; in the presence of .01M sodium sulfate the curve becomes even more sigmoid. Although the nature of the inhibition by sulfate is not clear, it is obviously quite different from the effect of arsenate.

Preliminary experiments show that the ionic concentration of the reaction medium has a profound effect on the kinetics of the reaction. For example, in the presence of 0.1M NaCl, the reaction follows

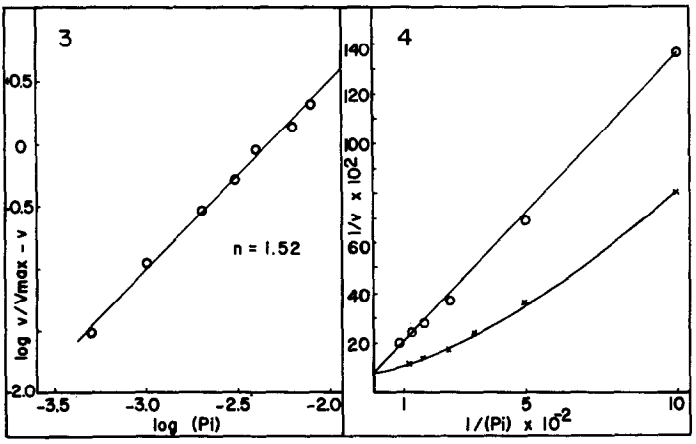

Figure 3. Data for the reaction in the absence of AMP, plotted according to equation 1.

Figure 4. Effects of arsenate on the reaction in the absence of AMP. X - X, no arsenate. O - O, .01M arsenate.

Michaelis-Menten kinetics even in the absence of AMP, with a Km of 15 x 10^{-3}M for Pi. AMP lowers the Km to about 5 x 10^{-3}M with no effect on Vmax.

Other results in our hands, which confirm the recent findings of Helmreich and Cori (1964) and Lowry, et. al. (1964), show that AMP decreases the Km and increases the Vmax with respect to glycogen, and that the Km for each substrate is dependent on the concentration of the other.

DISCUSSION

These data can be explained by assuming that there are at least 3 sites on the enzyme surface: an active site, a phosphate regulatory site, and an activator, or AMP, site. The phosphate regulatory site may or may not be catalytically active.

Phosphate, when bound to the regulatory site, would cause a conformational change in the enzyme which would increase the affinity of the active site for phosphate. This would give rise to a sigmoid

curve when plotting v vs. (Pi). AMP, when bound to its site, would increase the affinity of the active site for phosphate. In this case, however, the AMP effect would be so strong as to overshadow the phosphate cooperative effect.

Although other explanations are possible to account for the sigmoid curve, (see Sanwal, et. al. (1964); Webb (1963)), the effects of arsenate lend support to the above scheme. Arsenate, by binding at the regulatory site, would either cause an effect similar to phosphate, or at least block the phosphate activation. In either case the kinetics of the reaction would be normalized. At the same time, it would increase the Km for phosphate at the active site by direct competition.

An increase in ionic concentration, by causing molecular changes, would abolish the cooperation between the phosphate sites. It would also affect to some extent the action of the AMP site; this is suggested by the fact that in the presence of AMP, the Km for phosphate is increased 3-fold by 0.10M NaCl. The finding that the activation by phosphate is totally abolished under these conditions, but not the activation by AMP, constitutes strong evidence in favor of separate activation sites for AMP and phosphate.

The results of Lowry, et. al. (1964) and Helmreich and Cori (1964) differed from our results in that they did not find a sigmoid curve in experiments similar to these. The reasons are not clear, although there were several differences in the methods employed. It is possible, for example, that phosphorylase a made from phosphorylase b using phosphorylase b kinase does not show this effect, but that ageing of the preparation is necessary. Helmreich and Cori (1964), using phosphorylase a prepared in this way, found that AMP decreased the Km for phosphate; at saturating concentrations of phosphate, however, AMP had no effect. In our hands, phosphorylase a extracted from rabbit skeletal muscle is sensitive to AMP activation even at saturating levels of phosphate.

The behavior of phosphorylase a in these experiments resembles that of other allosteric enzymes, as discussed by Monod, et. al. (1963).

ACKNOWLEDGEMENTS

The author thanks Dr. James W. Green for his encouragement and aid in this work. Thanks are also due to Dr. Walter J. Nickerson for several helpful suggestions. This work was supported in part by Grant GM-T866 from U.S. Public Health Service.

REFERENCES

CORI, C.F. and CORI, G.T., Proc. Soc. Exp. Biol. Med., 34:702 (1936).

CORI, C.F. and CORI, G.T., Proc. Soc. Exp. Biol. Med., 36:119 (1937).

HELMREICH, E. and CORI, C.F., Proc. Nat. Acad. Sci., 51:131 (1964).

LOWRY, O.H., SCHULZ, D.W., AND PASSONNEAU, J.V., J. Biol. Chem., 239: 1947 (1964).

MADSEN, N.B., Biochem. Biophys. Res. Comm., 15:390 (1964).

MONOD, J., CHANGEUX, J.P., and JACOB, F., J. Mol. Biol., 6:306 (1964).

SANWAL, B.D., ZINK, M.W., and STACHOW, C.S., J. Biol. Chem., 239:1597 (1964).

WEBB, J.L., Enzyme and Metabolic Inhibitors, Vol. 1, p. 36, Acad. Press, New York (1963).