ALLOSTERIC PROPERTIES OF PHOSPHORYLASE b

Neil B. Madsen

Department of Biochemistry, University of Alberta,
Edmonton, Alberta

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The inhibition of phosphorylase b by ATP, first reported by Parmeggiani and Morgan (1962) and shown by them to be relieved by adenosine-5'-monophosphate (AMP), may be of considerable importance in controlling the activity of this enzyme in vivo. While investigating the kinetics of this phenomenon, we observed that AMP and glucose-1-phosphate (G-1-P), the activator and one of the substrates for the enzyme, have reciprocal effects on each other's K_m values; that is, an increase in the concentration of AMP results in a decrease in the K_m for G-1-P, while an increase in the concentration of G-1-P results in a decrease in the K_m for AMP. While this work was in progress, similar results were reported in a more extensive study by Helmreich and Cori (1964). They concentrated more on the other substrates for phosphorylase b, namely, inorganic phosphate and glycogen, and showed that an increase in the concentration of AMP caused a decrease in the K_m's for both of these two substrates, while an increase in the concentration of either inorganic phosphate or glycogen caused a decrease in the K_m for AMP.

The data we wish to report here confirm and complement the work of Helmreich and Cori. In addition, it is shown that the inhibition by ATP follows strictly competitive kinetics with respect to the concentration of AMP. Increasing the concentration of G-1-P at a constant level of AMP will also relieve the inhibition caused by ATP, but in this case, the

kinetics are not of the competitive type. Rather, in the presence of ATP, a plot of velocity versus substrate (G-1-P) concentration shows a sigmoid shape typical of other "allosteric" proteins and enzymes, as discussed by Monod, Changeux and Jacob (1963). This phenomenon lends support to the idea that phosphorylase b is an allosteric enzyme which can exhibit interaction between sites specific for substrates on the one hand and activators or inhibitors on the other.

METHODS AND MATERIALS

Phosphorylase b was prepared from rabbit muscle and recrystallized four times by the method of Fischer and Krebs (1962). Before use, it was transferred into 0.02 M glycerophosphate - 0.0015 M EDTA, pH 6.7, by passage through a column of Sephadex G-25. Immediately before each experiment, suitable aliquots of the stock solution were pre-incubated for 15 min. at 30° with 2% glycogen and .01 M cysteine. Enzymatic activity was determined by measuring the release of inorganic phosphate from G-1-P at 30° in reaction mixtures containing 0.5% rabbit liver glycogen, 0.01 M glycerophosphate, 0.0025 M cysteine, 0.0002 M EDTA, G-1-P and AMP as indicated, with all reagents adjusted to pH 6.7. All activities are expressed as micrograms of inorganic phosphate released in the first minute, calculated from the first order constants by the method of Cori, Cori and Green (1943). The validity of this procedure was checked by experiments which showed that first order kinetics were followed under the conditions reported here.

RESULTS

In some experiments, the enzymic activity was measured at various concentrations of both G-1-P and AMP and the resultant data were analysed by the use of reciprocal plots according to the procedure of Florini and Vestling (1957). Re-plotting of the V_{max} values for each set of data gave the theoretical K_m and V_{max} values for "infinite" AMP or "infinite" G-1-P concentrations. Table I shows the results of one such experiment.

As discussed above, the trend of the results agrees with those of Helmreich and Cori, although there are differences in the absolute values.

TABLE I

K_m OF AMP OR G-1-P AT DIFFERENT CONCENTRATIONS OF G-1-P OR AMP

Concentration of G-1-P, mMolar	V_{max}*	K_m of AMP M x 10^{-5}	Concentration of AMP, mMolar	V_{max}*	K_m of G-1-P M x 10^{-3}
3.0	2.17	12.0	0.04	1.85	7.4
7.5	2.57	6.3	0.2	3.44	5.1
15.0	2.95	5.3	1.0	3.85	5.1
24.0	3.45	4.5			
"infinite"	4.00	4.5	"infinite"	3.85	4.0

*Micrograms of inorganic phosphate per minute.

Fig. 1 shows the effect of 0.009 M ATP on the velocity of the enzymic reaction at increasing concentrations of G-1-P.

It is readily seen that in the presence of ATP these plots assume a sigmoid shape. The effect is increased when the AMP concentration is decreased. First order constants for the data in Fig. 1A remained constant

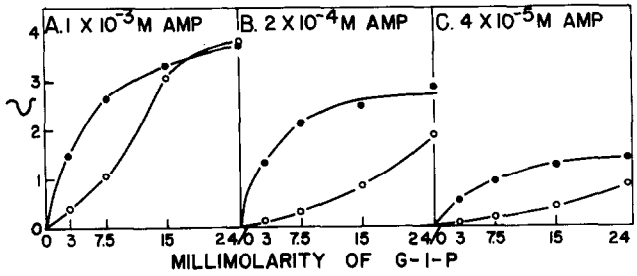

Figure 1. The velocity of the phosphorylase b reaction (micrograms of inorganic phosphate released per minute) at various concentrations of G-1-P and AMP. ● —— ●, no ATP, O —— O, 9 x 10^{-3} M ATP.

for samples taken at 5, 10, and 15 minutes of incubation. In Fig. 2A, the data from Fig. 1A are plotted according to the method of Lineweaver and Burk (1934), and it may be seen that the kinetics do not obey the normal competitive form.

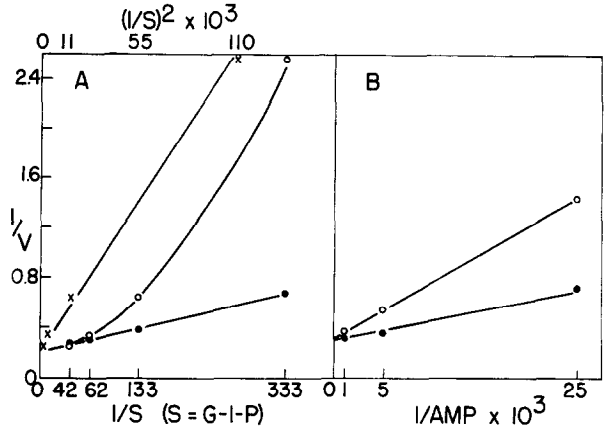

Figure 2, A. The inhibition of phosphorylase b by ATP at various concentrations of G-1-P. 1 x 10^{-3} M AMP was present. ● —— ●, no ATP; O —— O, 9 x 10^{-3} M ATP; X —— X, 1/v in the presence of 9 x 10^{-3} M ATP is plotted against the square of the reciprocal of the G-1-P concentration. B. The inhibition of phosphorylase b by ATP at various concentrations of AMP. 0.024 M G-1-P was present. ● —— ●, no ATP; O —— O, 9 x 10^{-3} M ATP.

However, if the reciprocal of velocity is plotted against the square of the reciprocal of substrate concentration, as was done for deoxythymidine kinase by Okazaki and Kornberg (1964), then the data follow a straight line. These authors state that such behaviour is suggestive of a bimolecular mechanism. Monod et al. (1963) showed that activity versus substrate relationships for allosteric enzymes obey the following form of Hill's empirical equation for the binding of oxygen to hemoglobin:

$$\log \frac{v}{V_{max} - v} = n \log S - \log K$$

The data presented here for the activity of phosphorylase b, in the presence of ATP, at various concentrations of G-1-P also form a straight line

when the above equation is applied and yield a value for n of 1.76. To quote Monod et al., this would "indicate cooperative interactions between homologous sites and also show that the reaction is not truly bimolecular."

DISCUSSION

Since the inhibition of phosphorylase b by ATP is competitive with respect to AMP, it is likely that ATP binds to the same site as does AMP. There are two binding sites for AMP on each molecule of the enzyme (Madsen and Cori, 1957). Presumably the way in which G-1-P relieves the inhibition is by increasing the affinity of the protein for AMP. The author has unpublished evidence that the number of binding sites for G-1-P equals that for AMP in the case of phosphorylase a, and if this is also true for phosphorylase b, then each protein molecule contains two sets of sites, each set capable of binding one molecule of AMP and one molecule of G-1-P. The data published by Helmreich and Cori (1964), together with that in this paper, leave little doubt that there is interaction between the AMP and G-1-P sites in each set. In addition, the present data suggest that in at least one condition, the inhibition by ATP, there is interaction between the two sets of sites on each protein molecule.

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