KINETIC MECHANISM OF SKELETAL MUSCLE CYCLIC AMP-DEPENDENT PROTEIN KINASE

Masafumi MATSUO, Ling CHANG, Ching-hsien HUANG and Carlos VILLAR-PALASI
Departments of Biochemistry and Pharmacology, University of Virginia, School of Medicine, Charlottesville, Virginia 22901, USA

Received 21 December 1977

1. Introduction

Despite the importance of cyclic AMP-dependent protein kinase in the hormonal regulation of metabolism in skeletal muscle [1], comparatively little is known about the reaction mechanism of this enzyme. We present here the results of initial velocity experiments and product inhibition studies on skeletal muscle cyclic AMP-dependent protein kinase, Peak I, or its isolated catalytic subunit. The results obtained are consistent with a random Bi Bi kinetic mechanism.

2. Experimental

Cyclic AMP-dependent protein kinase, peak I, was partially purified from rabbit skeletal muscle as in [2]. The holoenzymatic activity was stimulated 7–8-fold by cyclic AMP. The catalytic subunit, essentially homogeneous, was obtained from rabbit skeletal muscle as in [3]. Radioactive [γ-^{32}P]ATP was prepared as in [4]. Specific radioactivity was of the order of 1–3 X 10^9 cpm/μmol. Contaminating [^{32}P]P_i phosphate was less than 2% of the total radioactivity. Calf thymus histone Type IIA, ATP and ADP were purchased from Sigma.

Protein kinase activity was measured by the incorporation of radioactivity from [γ-^{32}P]ATP into histone [2]. Phosphohistone was prepared as in [5]. Phosphate content of the phosphohistone was determined as in [6,7]. ATP concentrations were determined by a coupled assay using glucose, hexokinase, glucose 6-P dehydrogenase and NADP, either spectrophotometrically at 340 nm, or fluorometrically [8].

3. Results

3.1. *Initial velocity studies*

Double reciprocal plots of initial velocity against the concentration of histone and Mg-ATP^{2-} at a series of fixed concentrations of the second substrate are illustrated in fig.1. A set of lines which intersect to the left of the vertical axis was obtained for each substrate when either the holoenzyme or its catalytic subunit was employed. This pattern is to be expected for an enzyme which catalyzes a 2 substrate reaction via a sequential rather than a ping–pong mechanism involving ternary enzyme–substrate complex [9]. The secondary plots of the intercepts and slopes of

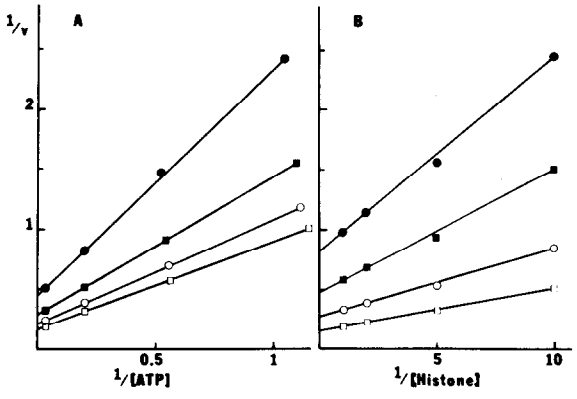

Fig.1. Double reciprocal plots of the reaction catalyzed by the holoenzyme. (1A) Plots of $1/v$ versus $1/[\text{Mg-ATP}^{2-}]$ at the following concentrations of histone: (•) 0.1 mg/ml; (▪) 0.2 mg/ml; (○) 0.5 mg/ml; (□) 1.0 mg/ml. (1B) Plots of $1/v$ versus 1/[histone] at the following concentrations of Mg-ATP^{2-}: (•) 1 μM; (▪) 2 μM; (○) 5 μM; (□) 30 μM.

Table 1
Values for the Michaelis and dissociation constants for the reaction catalyzed by cyclic AMP-dependent protein kinase and its catalytic subunit[a]

Kinetic constant	Holoenzyme	Catalytic subunit
K_m^A (μM)	6	10
K_m^B (mg/ml)	0.27	0.22
K_s^A (μM)	4	4

[a] A and B are Mg-ATP^{2-} and histone, respectively

Errors for the kinetic constant are about 10–15%

the reciprocal plots shown in fig.1 versus the reciprocal concentrations of the non-varied substrates were all linear and permited calculations of the parameters, K_m^A, K_m^B and K_s^A. The results are given in table 1.

3.2. *Product inhibition with Mg-ADP$^-$*

The initial rate experiments were also carried out for the cyclic AMP-dependent protein kinase and its catalytic subunit, respectively, in the presence of a single product, Mg-ADP$^-$. Figure 2 shows the kinetic patterns. In fig.2A, the plot of $1/v$ versus 1/(Mg-ATP^{2-}) at different fixed concentrations of Mg-ADP$^-$ clearly indicated that Mg-ADP$^-$ was competitive with respect to Mg-ATP^{2-} for the catalytic subunit. A similar pattern was also observed for the holoenzyme. Figure 2B shows that Mg-ADP$^-$ functions as a linear non-competitive inhibitor for histone phosphorylation. The fact that Mg-ADP$^-$ does not exhibit a mixed-type inhibition pattern with respect to histone can be taken as additional evidence to rule out ping–pong Bi Bi reaction mechanisms for this protein kinase. Secondary plots of the slopes of the lines of fig.2A and of the similar plot obtained for the holoenzyme against the concentration of Mg-ADP$^-$ indicate a linear relationship. The apparent dissociation constant, K_i, for the enzyme–inhibitor complex can be calculated from the horizontal intercept of these secondary plots. A value of 15 μM was obtained for the holoenzyme at fixed histone conc. 0.5 mg/ml, while 17 μM was calculated for the catalytic subunit at 0.55 mg/ml of fixed histone concentrations.

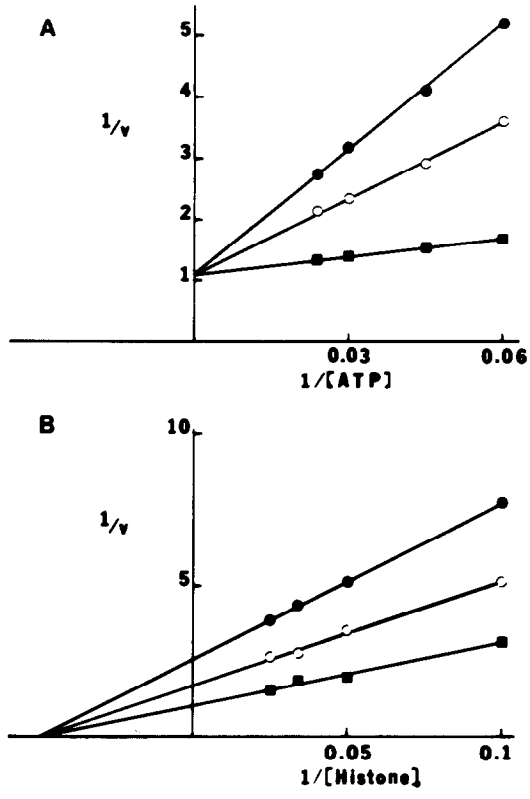

Fig.2. Double reciprocal plots of the inhibition of catalytic subunit by Mg-ADP$^-$. (2A) Plots of $1/v$ versus 1/[Mg-ATP^{2-}] at a fixed histone concentration of 0.55 mg/ml. (■) No additions; (○) 52.5 μM Mg-ADP$^-$; (●) 125 μM Mg-ADP$^-$. (2B) Plots of $1/v$ versus 1/[histone] at a fixed Mg-ATP^{2-} concentration of 33 μM. (■) No additions; (○) 62.5 μM Mg-ADP$^-$; (●) 125 μM Mg-ADP$^-$.

3.3. *Product inhibition with phosphohistone*

In order to determine whether the sequential mechanism of the protein kinase catalyzed reaction is random or ordered, the initial rate experiments in the presence of the second product, phosphohistone, were carried out. It is anticipated that a non-competitive inhibition pattern of phosphohistone with respect to histone would be observed for an ordered Bi Bi or *iso* ordered Bi Bi mechanism, and that a competitive inhibition pattern would be displayed for a random Bi Bi mechanism [10]. Phosphohistone shows competitive inhibition with respect to histone, with an apparent dissociation constant, K_i, of 0.4 mg/ml at fixed conc. 3 μM ATP. This finding,

together with other data presented earlier, suggest strongly that the random Bi Bi mechanism is most compatible with the reaction scheme for cyclic AMP-dependent protein kinase.

4. Discussion

The results presented are consistent with a random Bi Bi reaction mechanism for either skeletal muscle cyclic AMP-dependent protein kinase or the free catalytic subunit. This mechanism is further corroborated by the observation that polyarginine is a competitive inhibitor of the enzyme with respect to histone, but a non-competitive inhibitor with respect to $Mg\text{-}ATP^{2-}$ [11]. In a number of protein substrates of skeletal muscle cyclic AMP-dependent protein kinase, including histone, the phosphorylation sites have been found in the primary amino acid sequences having the general structure –Arg–X–Y–Ser(P)– [12]. Presumably, this vicinal arginyl residue is important for enzyme–substrate association. Accordingly, polyarginine can be considered as a dead-end inhibitor which combines with the enzyme. For a random Bi Bi reaction mechanism, a dead-end inhibitor which is competitive with respect to one substrate generally shows non-competitive inhibition with respect to the other substrate [10]. The inhibition patterns of polyarginine are indeed compatible with the proposed random Bi Bi reaction mechanism.

Random sequential kinetics for the phosphorylation of a number of synthetic substrates by a calf thymus protein kinase have reported [13]. Similar kinetics have been proposed for a *Neurospora crassa* protein kinase using casein as a protein substrate [14]. This investigation, however, presents the first kinetic studies on the reaction mechanisms of phosphorylation of histone mixtures by a skeletal muscle cyclic AMP-dependent protein kinase (type I) and its catalytic subunit.

Acknowledgements

We thank Dr Laura Huang for kindly providing the cyclic AMP-dependent protein kinase. This investigation was supported by USPHS Grant GM-22430 to C. H. and by AM-14466 to C. V.-P.

References

[1] Nimmo, H. G. and Cohen, P. (1977) in: Adv. Cyclic Nucleotide Res. (Greengard, P. and Robinson, G. A. eds) vol. 8, pp. 145–266.
[2] Huang, L. C. and Huang, C. (1975) Biochemistry 14, 18–24.
[3] Betchel, P. J., Beavo, J. A. and Krebs, E. G. (1977) J. Biol. Chem. 252, 2691–2697.
[4] Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. 90, 147–149.
[5] Meisler, M. H. and Langan, T. A. (1969) J. Biol. Chem. 244, 4961–4968.
[6] Ames, B. N. (1966) Clin. Chim. Acta 14, 361–366.
[7] Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361–366.
[8] Williamson, J. R. and Corkey, B. E. (1969) in: Methods in Enzymology (Lowenstein, J. M. ed) vol. 13, pp. 434–513, Academic Press, New York.
[9] Cleland, W. W. (1970) in: The Enzymes, (Boyer, P. D. ed) vol. 2, pp. 1–65, Academic Press, New York.
[10] Fromm, H. J. (1975) Initial Rate Enzyme Kinetics, Springer-Verlag, New York.
[11] Matsuo, M., Huang, C. and Huang, L. C. (1978) in preparation.
[12] Cohen, P., Rylatt, D. B. and Nimmo, G. A. (1977) FEBS Lett. 76, 182–186.
[13] Pomerantz, A. H., Allfrey, V. G., Merrifield, R. B. and Johnson, E. M. (1977) Proc. Natl. Acad. Sci. USA 74, 4261–4265.
[14] Gold, M. H. and Segel, I. H. (1974) J. Biol. Chem. 249, 2417–2423.