Bioorganic & Medicinal Chemistry Letters, Vol.1, No.4, pp. 193-196, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

Inositol Dependent Phosphate-Oxygen Ligand Exchange Catalysed by Inositol Monophosphatase.

Graham R. Baker and David Gani*

Chemistry Department, The Purdie Building, The University, St. Andrews, Fife. KY16 9ST, U.K.

(*Received* 15 *March* 1991)

Summary: Inositol monophosphatase is unable to catalyse the exchange of ^{18}O-label from ^{18}O-water into inorganic phosphate in the absence of inositol. The inositol mediated exchange reaction is Mg^{2+} dependent, shows a similar pH dependence to that for the hydrolysis of *myo*-inositol 1-phosphate and is inhibited by lithium cation. These results suggest that the enzyme operates *via* a ternary complex mechanism unlike alkaline and acid phosphatase.

Aspects of the metabolic pathways associated with the release of calcium from intracellular stores during signalling processes have been defined recently.[1] An important and key step involves the hydrolysis of inositol 1-phosphate by the Mg^{2+}-dependent lithium-sensitive phosphoesterase, inositol monophosphatase, to give free inositol.[2] Free inositol is recycled to provide the precursor, phosphatidylinositol 4,5-bisphosphate, for the two secondary messengers, diacylglycerol and inositol 1,4,5-trisphosphate.

Based on the observation that the rate of [^{14}C]-inositol formation from [^{14}C]-inositol 1-phosphate at high [Li^+] was faster than the steady state rate for substrate turnover, together with the established uncompetitive mode of inhibition by Li^+, we recently suggested that bovine brain inositol monophosphatase might operate *via* a substituted enzyme mechanism in which the phosphate group of the substrate was first transferred to an enzyme bound nucleophile, and in a subsequent step to water.[3] We further suggested that Li^+ might act by retarding the rate of conversion of the phosphoryl enzyme to free enzyme, relative to the rate of phosphorylated enzyme formation.

During these studies it was noted that P_i acted as a competitive inhibitor, K_i = 0.5 mM at pH 7.8, confirming that P_i is the second product off, while the other product inositol, had no effect on the rate of inositol 1-phosphate hydrolysis at concentrations of up to 50 mM. Furthermore, in incubations containing [^{14}C]-inositol and *unlabelled* Inositol 1-phosphate, or, the alternative

substrate adenosine 2'-phosphate (2'-AMP), transphosphorylation to give [^{14}C]-inositol phosphate was not detected.[4] The lack of an effect by inositol in each experiment was taken to indicate that inositol possessed a high K_i value although further analysis was obviously precluded. Since the sequence of events following the formation of the putative phosphoryl enzyme still needed to be defined, the ability of the enzyme to catalyse the incorporation of ^{18}O-label from [^{18}O]-water into the second product, P_i, was assessed.

Accordingly, several identical incubation solutions containing homogeneous bovine brain inositol monophosphatase,[2,4] potassium dihydrogen phosphate (20 mM), magnesium chloride (2 mM) and Tris acetate buffer (50 mM) in a total volume of 250 µl of 7 % [^{18}O]-water, were prepared. Controls contained no enzyme. The reaction solutions were kept at 37 °C and at time intervals over a period of several hours individual incubations, in duplicate, were quenched by cooling in liquid nitrogen. The frozen solutions were lyophilised and the residues were redissolved in $MeOH/HCl_{(aq)}$ (98:2). Excess ethereal diazomethane was added, the unreacted diazomethane was removed in a stream of dintrogen and the particulate material was removed by centrifugation. The supernatent solution was reduced in volume *in vacuo* and was then subjected to analysis by GC/MS under conditions previously optimised using authentic samples of trimethyl phosphate.

The mass spectrum for the trimethyl phosphate isolated from the control reactions and from the incubations quenched at t = 0 showed the expected parent ion at 140 amu. However, the samples prepared from incubations quenched after up to 8 hours showed no significant ^{18}O-enrichment, as judged by the intensity of the ion at 142 amu, where a 0.25% incorporation would have been detected easily.

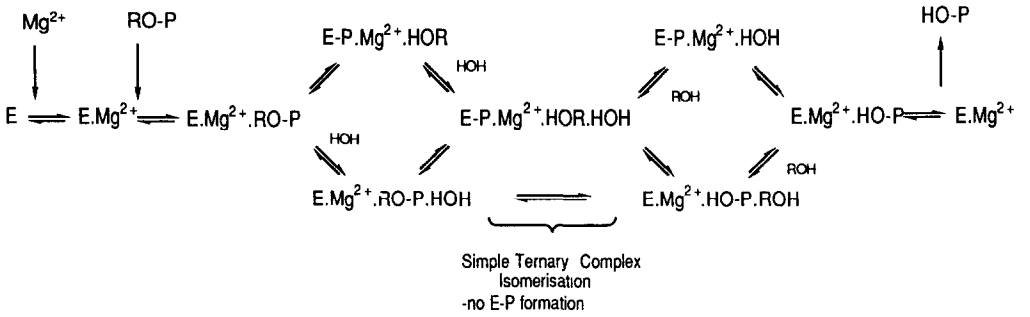
This was a surprising result in the light of similar studies performed with other phosphatases in which facile ^{18}O-label incorporation was observed.[5,6]

In an attempt to maximise the opportunity for ^{18}O-label incorporation, the entire procedure was repeated using more enzyme and higher proportions of labelled water for each of a range of alternative conditions where the pH of the incubation medium and [P_i] was varied. No incorporation of ^{18}O-label was observed.

Enzyme phosphorylation through reverse steps had been demonstrated for alkaline[5] and acid phosphatase.[6] Therefore, the lack of phosphate ligand exchange for inositol monophosphatase appeared to indicate that, upon the formation of the putative phosphorylated enzyme, E-P, the leaving [^{16}O]-water molecule could not debind, in order to allow [^{18}O]-water to bind, before it re-attacked E-P. For a substituted enzyme mechanism where inositol and water share a common (or overlapping) binding site, inositol must debind before water can bind to the enzyme, in the forward reaction direction. In this situation, where the second substrate (water) binds after the first product (inositol) is released, it would be anticipated that inositol could have no effect in promoting the incorporation of ^{18}O-label into phosphate in experiments of the type described

above. On the other hand, if water was able to bind to the enzyme before inositol was released from its site, the expected result would be different. The microscopic reverse would require that the inositol must bind before the water, derived from P_i, could be released from the enzyme to equilibrate with the solvent. Such a mechanism would predict that inositol would facilitate the incorporation of ^{18}O-label into phosphate.

In order to test this possibility, experiments of the type outlined above were performed in the presence of 20 mM inositol. Analysis of the phosphate samples as the ester derivatives, *vide supra*, indicated that time dependent ^{18}O-label incorporation did occur. Thus, inositol monophosphatase was able to bind to both phosphoryl group acceptors simultaneously, Scheme 1.



Scheme 1

Further experiments performed over the pH range 5.0 to 9.0, in 0.5 pH unit increments, demonstrated that the optimum pH for the inositol dependent exchange reaction was ~8.0, similar to the optimum pH for the phosphoesterase activity.[4,7] Experiments in which Mg^{2+} was omitted showed no activity whatsoever, revealing that Mg^{2+}, as for the physiological reaction, was essential for the phosphate-oxygen ligand exchange reaction. It was also established that the exchange reaction was inhibited by Li^+. The IC_{50} value of 1.0 mM was in the expected range based on the values of K_i obtained for the phosphoesteratic hydrolyses of inositol 1- and 4-monophosphates reported by us and by others.[2,4,7]

By conducting a series of time course experiments at 20 mM phosphate (~40 times K_i for product inhibition *i.e.* saturating [P_i]) and at five different concentrations of inositol (from 5 to 100 mM), an apparent K_m of 195±20 mM was obtained for inositol for the exchange reaction. This value corresponds to the lower limit of the K_i value for inositol product inhibition and explains why no significant inhibition of the physiological reaction by inositol was detected.

Extrapolation of the double reciprocal plot for the $v_{Exchange}$ versus [inositol] at 20 mM phosphate gave a V_{max} value close to (~60% of) that for the physiological reaction indicating that the rate of phosphoryl transfer from water is about as fast as the slowest step in the forward reaction direction. Thus, if the two reactions are limited by a common step or steps, which seems quite

probable, the inositol desorption step (in addition to those for phosphoryl transfer to water and the desorption of P_i)[3] must now also be considered as a likely contender .

In conclusion, we have demonstrated that inositol monophosphatase differs from other phosphatases in its ability to bind, simultaneously, both phosphoryl group acceptor molecules, inositol and water, to form a ternary complex. Furthermore, we have shown that the inositol dependent phosphate-oxygen ligand exchange reaction displays the same cofactor requirements, pH-dependence and Li^+ sensitivity as the physiological reaction. The results of this study rule-out the operation of a ping-pong mechanism[3] where *free* E-P.Mg^{2+} is formed (not shown in Scheme 1) but are consistent with a *substituted* ternary complex mechanism where E-P.Mg^{2+}.HOR.HOH is formed, Scheme 1. Since the operation of a simple (non-substituted) ternary complex mechanism (lower part of Scheme 1) cannot be excluded on the basis of the results described here and since a free hydroxyl group (adjacent to the phosphate ester) in the substrate is required for catalysis (*cf.* ribonuclease), further annotation of the catalytic process must rely on stereochemical studies using phosphorothioate substrates.[8] Nevertheless, all previous studies of phosphohydrolase reactions involving alcoholic nucleofuges, with the exception of that for 5'-nucleotidase,[9] have shown that phosphoryl transfer occurs in two steps *via* a phosphorylenzyme intermediate.[10]

Acknowledgements

We thank the SERC and Merck, Sharp and Dohme for a CASE studentship to G.R.B. and for financial support and Dr. D. Corina for mass spectrometric analysis of the trimethyl phosphate samples. Some of the work described here was performed in the chemistry department at Southampton University.

References

1. For a recent review see Proceedings of the 627th Meeting of the Biochemical Society, *Biochem. Soc. Trans.* **1989**, *17*, 1.
2. Gee, N. S.; Reagan, C. I.; Watling, K. J.; Aspley, S.; Jackson, R. G.; Reed, G. G.; Gani, D.; Shute, J. K. *Biochem. J.* **1988**, *249*, 883.
3. Shute, J. K.; Baker, R.; Billington, D.C.; Gani, D. *J. Chem. Soc., Chem. Comm.* **1988**, 626.
4. Shute, J. K.; Baker, G. R.; Cohen, M. A.; Leech A. P.; Gani, D in preparation.
5. VanEtten, R. L.; Risley, J. M. *Proc. Natl. Acad. Sci. USA*, **1978**, *75*, 4784
6. Schwartz, J. H. *Proc. Natl. Acad. Sci. USA*, **1963**, *49*, 871.
7. Attwood, P. V.; Ducep, J-B.; Chanel, M-C. *Biochem. J.* **1988**, *253*, 387.
8. Baker, G. R.; Billington, D. C.; Gani, D. *Biomed. Chem Lett.* **1991**, *1*, 17.
9. Tsai, M-D. *Biochemistry*, 1980, **19**, 5310.
10. See Knowles, J. R. *Ann. Rev. Biochem.*, **1980**, *49*, 887, and;. Floss, H. G.; Tsai, M-D.; Woodard, R. W *Topics Stereochem.*, **1984**, *15*, 253 for reviews.