Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 513-519

NUCLEOTIDE ACTIVATION OF GLYCOGEN PHOSPHORYLASE b OCCURS ONLY WHEN THE NUCLEOTIDE PHOSPHATE IS IN A DIANIONIC FORM

Stephen G. Withers and Neil B. Madsen

Department of Biochemistry,
University of Alberta,
Edmonton, Alberta,
Canada T6G 2H7

Received October 13,1980

SUMMARY. Adenosine monophosphofluoridate has been synthesised and purified to remove all contaminating AMP. This AMP analogue fails to activate glycogen phosphorylase b, even at high concentration, but inhibits the AMP activation with a Ki value of 3 mM. Activation of phosphorylase b by adenosine phosphoramidate has been re-investigated in the light of these findings and a purified sample of this nucleotide analogue has been shown to produce little or no activation of the enzyme. These findings are interpreted in terms of an absolute requirement of the nucleotide activatorsite in phosphorylase for a nucleotide with a dianionic phosphate. The implications of this for the role of the phosphate moiety in the proposed mechanism of activation are discussed.

Glycogen phosphorylase b shows an absolute requirement for AMP for catalytic activity while phosphorylase a is active in its absence, but can be activated a further 20% by binding of this nucleotide. AMP, however, binds to phosphorylase a an order of magnitude more tightly than to phosphorylase b.

Many experiments have been performed with numerous nucleotide analogues to determine the binding and activation specificity of phosphorylase b for nucleotide (1,2,3,4,5,6). From these studies it was concluded that the 5'-phosphate moiety of the nucleotide is critically required for the activation while the precise structure of the nucleoside moiety was less critical. However, both the phosphate and the purine ring are involved in binding. Of the AMP analogues modified in the 5'-phosphate, only adenosine 5'-phosphorothioate and adenosine 5'-phosphoramidate showed activation of the enzyme. In the case of adenosine 5'-phosphorothioate (7), which would be dianionic under these experimental conditions, (pKa = 5.3 (8)) activation parameters were similar to those exhibited by AMP. Adenosine 5'-phosphoramidate, which is monoanionic, showed much weaker

Abbreviations: AMPF = adenosine 5'-phosphofluoridate; DTT = dithiothreitol.

0006-291X/80/220513-07$01.00/0

binding (Ka = 3 mM) and Vmax values up to 76% of that shown by AMP (1,2,6). It thus appeared that while a 5'-phosphate group was essential for activation, it need not be dianionic. Indeed, it was suggested (2) that a hydrogen was required on the nucleotide phosphate group for activation of phosphorylase b. However, ^{31}P NMR studies of adenosine 5'-phosphorothioate bound to phosphorylase indicate that it is bound in a dianionic form (9,10). Recent assignments of arginine residues at the AMP binding site of phosphorylase a and b by chemical techniques (11,12) and the X-ray crystallographic identification of three arginine residues, Arg 308, Arg 309 and Arg 242 at the phosphate binding pocket to chelate the phosphoryl group (13) all indicate a highly positively charged binding pocket. This implies a binding site more suited to a dianionic than a monoanionic phosphate.

In order to investigate this requirement, we have synthesised adenosine 5'-monophosphofluoridate. This compound has only a single negative charge on its phosphate group and thus provides an excellent analogue for testing the importance of the overall charge of the nucleotide phosphate in the activation of the enzyme. Since the fluorine atom is smaller than the hydroxyl group (Van der Waals radii equal 1.35 Å and 2.20 Å respectively), binding of this nucleotide should not be hindered sterically: differences in activation properties can then be assigned to charge differences. The apparent conflict between the experiments described and those performed previously with adenosine 5'-phosphoramidate has been investigated by purification of the phosphoramidate and redetermination of its activation properties. The results of these latter experiments are also described.

MATERIALS AND METHODS

Rabbit muscle glycogen phosphorylase b was prepared by the method of Fischer and Krebs (14) using DTT instead of cysteine, and recrystallised at least three times before use. Phosphorylase a was prepared from phosphorylase b with phosphorylase kinase (EC 2.7.1.38) (15). Initial reaction rates were determined at 30°C by the Fiske-Subbarow phosphate analysis in the direction of saccharide synthesis as described by Engers et al. (16). The buffer used in all kinetic experiments was 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM DTT, 1 mM EDTA adjusted to pH 6.8.

AMP, adenosine 5'-phosphoramidate and buffer chemicals were obtained from Sigma Chemical Co. except for DTT which was obtained from Bio-Rad Laboratories.

Fluorodinitrobenzene was obtained from Eastman Kodak Co. and all other chemicals from Fisher Chemical Co.

Adenosine 5'-phosphofluoridate was prepared by the method of Wittman (17) and was purified by gradient elution (10 mM/100 mM) with ammonium bicarbonate buffer from a DE52 ion exchange column in the carbonate form at pH 8. The freeze dried ammonium salt was used for kinetic experiments. Adenosine 5'-phosphoramidate was purified by the same method. Thin layer chromatography was performed in the ascending mode on Macherey Nagel Polygram CEL 300 UV_{254} cellulose plates in the solvent system isopropyl alcohol (7): ammonium hydroxide (1): water (2).

RESULTS

Purified adenosine 5'-phosphofluoridate was tested as an activator of phosphorylase b at concentrations ranging from 0.045 mM to 10 mM using 24 mM glucose-1-phosphate. No activity of the enzyme could be detected under these conditions, whereas AMP activated with a Ka of 0.04 mM (2). AMPF was also tested as an inhibitor of the AMP activation of phosphorylase b . The data plotted by the Lineweaver Burk method yielded curved lines which converged on a common value for 1/Vmax, suggesting competitive inhibition and positive homotropic cooperativity between AMP binding sites. Similar inhibition patterns were observed with ATP (18), which binds to the AMP site (19). Our data for AMPF yielded the Hill plot shown in Fig. 1. A replot of the apparent Km's versus inhibitor concentrations gave an apparent Ki of 2.7 mM. Thus AMPF binds weakly causing inhibition, but cannot activate the enzyme. It does not, however, irreversibly inhibit the enzyme since control experiments of enzyme incubated with AMPF showed no time-dependent loss of activity.

Experiments performed with phosphorylase a in the absence of AMP at varying concentrations (0.5 mM to 10 mM) of AMPF showed that some activation of phosphorylase a was observed at low concentrations of AMPF, but increasing concentrations of AMPF caused inhibition of the enzyme to rates below that observed for phosphorylase a in the absence of AMP. This is probably explained by the presence of a very low concentration (0.1% ≡ 1 μM) of contaminating AMP. This would be sufficient to provide partial activation of phosphorylase a (Ka = 2 μM) but not of phosphorylase b (Ka = 40 μM). At higher analogue concentrations, the inhibitory effect dominates, reversing the activation and giving activities

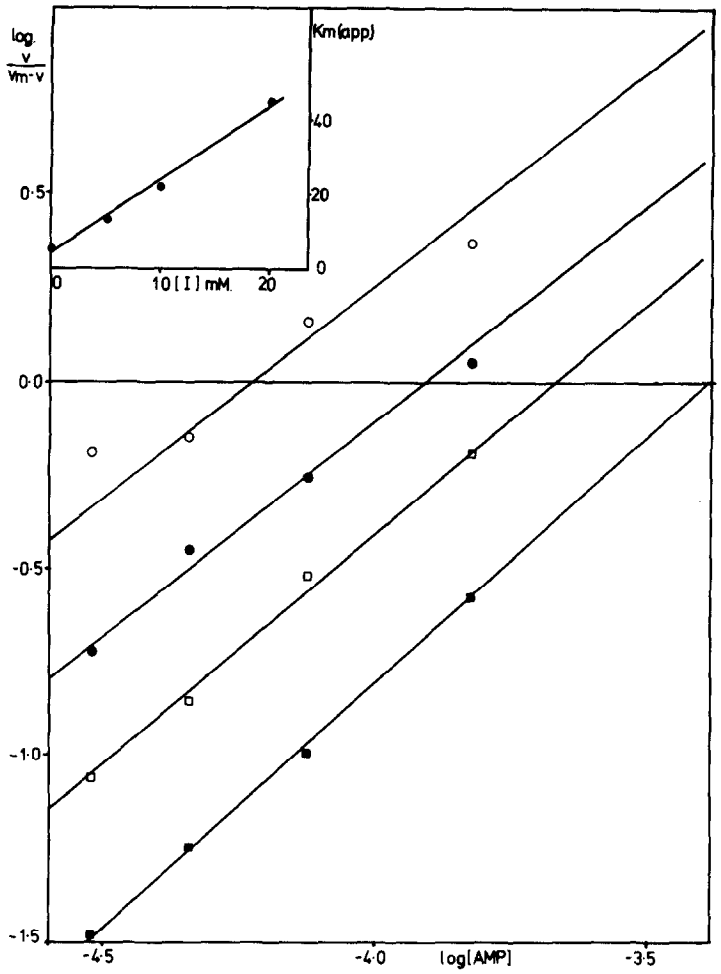

Figure 1: Hill plot for inhibition of phosphorylase b by AMPF at varying AMP concentrations. Concentrations of AMPF used were (o) 0 mM, (●) 5 mM, (□) 10 mM, (■) 20 mM.
Inset: Replot of apparent Km versus inhibitor concentration.

lower than control. Control experiments using 1 μM AMP demonstrated that phosphorylase a was activated, but phosphorylase b remained essentially inactive. This may indicate that AMPF inhibits phosphorylase a by binding at the nucleoside (20) site rather than the nucleotide site since only by this type of inhibition would activities lower than control be observed.

Commercial adenosine 5'-phosphoramidate was investigated as an activator of phosphorylase b and results were obtained which were essentially identical to those reported previously (1,2,6) with Ka ≃ 2 mM, Vm = 50 μM/min/mg.

Adenosine 5'-phosphoramidate was purified as described in Materials and Methods, and contaminating AMP removed. The amount of AMP contaminant isolated was determined by UV analysis and found to constitute 3% w/w of the original sample. This purified adenosine 5'-phosphoramidate was then tested as an activator of phosphorylase b and found to have a drastically reduced activity (Vm = 4 μM/min/mg) though not a complete lack of activity. The purified material was therefore investigated by thin layer chromatography as described in Materials and Methods and found to still contain a small amount of contaminating AMP. This purified material was then repurified by the original procedure and a small amount (0.3%) of AMP contaminant removed. Kinetic experiments with this repurified material, however, still showed similar activities but thin layer chromatographic analysis of this sample revealed the presence of more AMP. This AMP probably arises from spontaneous hydrolysis of the phosphoramidate to the ammonium phosphate salt. Similar hydrolysis behavior has been observed previously with phosphoramidic acid in aqueous solution at pH 7 (21).

CONCLUSIONS

The total lack of activity of phosphorylase b in the presence of high concentrations of adenosine 5'-phosphofluoridate indicates that the activation process has an absolute requirement for a dianionic phosphate moiety attached to the adenosine. The results indicating greatly diminished activity with purified adenosine 5'-phosphoramidate also support this hypothesis, as do the results obtained previously (7) with adenosine 5'-phosphorothioate, which is the only nucleotide modified in the phosphate moiety capable of activating rabbit muscle glycogen phosphorylase b. AMPF can, however, bind, as shown by its inhibition of AMP activation. The pattern of its inhibition of phosphorylase b strongly suggests that it binds at the nucleotide site. The results with phosphorylase a indicate that AMPF may be inhibiting by binding at the nucleoside site since even in the presence of small amounts of contaminating AMP, the compound can inhibit the action of the enzyme to values lower than

those observed in the absence of AMP. Such inhibition could not be caused by binding at the nucleotide site.

No irreversible inactivation of the enzyme was observed upon prolonged incubation of the enzyme with the inhibitior as might have been the case if it behaved as diisopropyl fluorophosphate does toward many esterases. This is not, however, surprising, since experiments on other enzymes with several other phosphofluoridates have failed to show any irreversible inactivation (22).

The array of arginine residues at the nucleotide site observed by X-ray crystallography indicates a tight phosphate binding site well suited to a dianionic species, and a molecular mechanism of activation has been proposed (23) on the basis of this data. This mechanism requires the phosphate moiety to act as an anchor point in the binding of the nucleotide. Once anchored, the Tyr-75 residue of the protein moves closer to interact and stack with the adenine ring and it is this movement that causes the allosteric conformational changes throughout the molecule. These movements have been observed by X-ray crystallographic analysis (24) of the activated and inhibited phosphorylase a. Such a mechanism requires extremely tight binding of the phosphate moiety, and the results described here would fit this theory very well. Presumably the lack of activation by monoanionic nucleotides arises because the phosphate cannot anchor itself tightly enough to cause the appropriate conformation changes. The interaction of the adenine ring with Tyr-75 has been observed in the tetragonal crystals of phosphorylase b (19). This mechanism is also supported by previous work (25) on 8-[m-(m-fluorosulfonyl benzamido)benzylthio]adenine which binds covalently to phosphorylase b at the nucleotide site leaving the enzyme permanently activated and with no requirement for AMP. In this case, the covalent bond formed between the nucleoside and the protein serves as the anchor point in the absence of a phosphate group. Similar enzymic conformational changes can then occur to those observed with AMP.

An absolute requirement for a doubly charged nucleotide phosphate for activation of phosphorylase is thus established and rationalised. Further con-

firmation of the proposed mechanism of nucleotide activation awaits a more detailed X-ray crystallographic analysis of both the activated and inhibited forms of the enzyme.

ACKNOWLEDGEMENTS

We wish to thank K. Boodram, L. Glew and S. Shechosky for their technical assistance and the Medical Research Council of Canada for financial support.

REFERENCES

1. Okazaki, T., Nakazawa, A. and Hayaishi, O. (1968) J. Biol. Chem. 243, 5266-5271.
2. Mott, D.M. and Bieber, A.L. (1970) J. Biol. Chem. 245, 4058-4066.
3. Black, W.J. and Wang, J.H. (1968) J. Biol. Chem. 243, 5892-5898.
4. Morange, M., Garcia-Blanco, F., Vandenbunder, B. and Buc, H. (1976) Eur. J. Biochem. 63, 553-563.
5. Graves, D.J. and Wang, J.H. (1972) The Enzymes 7, 435-482.
6. Black, W.J. and Wang, J.H. (1970) Biochim. Biophys. Acta 212, 257-268.
7. Murray, A.W. and Atkinson, M.R. (1968) Biochemistry 7, 4023-4029.
8. Jaffe, E.K. and Cohn, M. (1978) Biochemistry 17, 652-657.
9. Feldmann, K. and Hull, W.E. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 856-860.
10. Withers, S.G., Sykes, B.D., Madsen, N.B. and Kasvinsky, P.J. (1979) Biochemistry 18, 5342-5348.
11. Li, E.C., Fletterick, R.J., Sygusch, J. and Madsen, N.B. (1977) Can. J. Biochem. 55, 465-473.
12. Dreyfus, M., Vandenbunder, B. and Buc, H. (1980) Biochemistry, in press.
13. Fletterick, R.J. and Madsen, N.B. (1980) Ann. Rev. Biochem. 49, 31-61.
14. Fischer, E.H. and Krebs, E.G. (1962) Methods Enzymol. 5, 369-373.
15. Krebs, E.G., Love, D.S., Bratvold, G.E., Trayser, K.A., Meyer, W.L. and Fischer, E.H. (1964) Biochemistry 3, 1022-1033.
16. Engers, H.D., Shechosky, S. and Madsen, N.B. (1970) Can. J. Biochem. 48, 746-754.
17. Wittmann, R. (1963) Chem. Ber. 96, 771-779.
18. Madsen, N.B. and Shechosky, S. (1967) J. Biol. Chem. 242, 3301-3307.
19. Johnson, L.N., Stura, E.A., Wilson, K., Sansom, S. and Weber, I.T. (1979) J. Mol. Biol. 134, 639-653.
20. Kasvinsky, P.J., Madsen, N.B., Sygusch, J. and Fletterick, R.J. (1978) J. Biol. Chem. 253, 3343-3351.
21. Changley, J.D. and Feageson, E. (1963) J. Amer. Chem. Soc. 85, 1181-1190.
22. Yount, R.G. (1975) Adv. Enzymol. 43, 1-56.
23. Fletterick, R.J., Sprang, S. and Madsen, N.B. (1979) Can. J. Biochem. 57, 789-797.
24. Madsen, N.B., Kasvinsky, P.J. and Fletterick, R.J. (1978) J. Biol. Chem. 253, 9097-9101.
25. Anderson, R.A., Parrish, R.F. and Graves, D.J. (1973) Biochemistry 12, 1901-1906.