Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 415-422

EFFECT OF POLYMYXINS ON GLYCOGEN PHOSPHORYLASE

T.B. Ktenas, N.G. Oikonomakos, T.G. Sotiroudis, S.I. Nikolaropoulos, and A.E. Evangelopoulos

The National Hellenic Research Foundation, 48 Vassileos Constantinou Ave., Athens 501/1, Greece

Received September 27,1980

SUMMARY

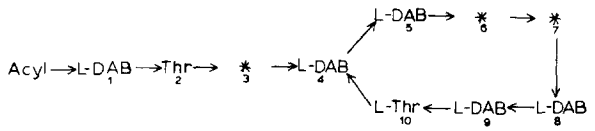
AMP-dependent activity of glycogen phosphorylase b is stimulated by the polymyxins A, B, D, and E. Kinetic studies indicate that these cyclic peptide antibiotics at low concentrations greatly enhance AMP-activation of the enzyme. The presence of polymyxins in the assay system leads to (a) partial desensitization of allosteric interactions toward AMP, (b) lowering of K_m for the substrates glucose-1-phosphate and glycogen, and (c) reversal of the glucose-6-phosphate inhibition. In contrast to phosphorylase b, neither AMP-phosphorylase b' system nor phosphorylase a (with or without AMP) is considerably activated by polymyxins.

INTRODUCTION

Glycogen phosphorylase b (EC 2.4.1.1) shows an absolute requirement for AMP for activity and is allosterically inhibited by ADP, ATP, and glucose-6-phosphate. Phosphorylation of Ser-14 of phosphorylase b by the enzyme phosphorylase kinase leads to phosphorylase a, an enzyme species which no longer requires AMP for activity and is not subject to allosteric inhibition (1). A useful variant of phosphorylase can be derived from phosphorylase a by limited proteolysis with trypsin. The phosphorylase b' produced is similar to phosphorylase b in that it requires AMP for activity (1).

It is well-established that AMP-activation of phosphorylase b is stimulated by several polycationic molecules (protamine, synthetic polylysine, polyamines), substrate anions, fluorides, and divalent metal ions (1). A phosphopeptide representing a segment of the NH_2-terminal region of phosphorylase a (2), phenothiazines, polycarboxyl-

0006-291X/80/220415-08$01.00/0



Polymyxin	Composition of variable regions			
	ACYL	3	6	7
Polymyxin A (M)	MOA	D-DAB	D-Leu	L-Thr
Polymyxin B_1	MOA	L-DAB	D-Phe	L-Leu
Polymyxin B_2	IOA	L-DAB	D-Phe	L-Leu
Polymyxin D_1	MOA	D-Ser	D-Leu	L-Thr
Polymyxin E_1	MOA	L-DAB	D-Leu	L-Leu
Polymyxin E_2	IOA	L-DAB	D-Leu	L-Leu

Diagram 1. Structures of the polymyxins. DAB, 2,4-diaminobutyric acid; MOA, 6-methyloctanoic acid; IOA, 6-methylheptanoic acid. The composition data were taken from (4).

ates, sulfate anions, and sulfated polysaccharides (3) were also reported as stimulators of AMP-activation of phosphorylase b.

The polymyxins belong to the group of peptide antibiotics, and affect a wide variety of biochemical processes in bacteria (4). They are characterized by a heptapeptide ring, a high percentage of 2,4-diaminobutyric acid, and a fatty acid attached to the peptide through an amide bond (Diagram 1). In addition to the two variable amino acid positions of the heptapeptide ring, a third variable position is also found in the peptide side chain. In view of their chemistry as being cyclic polycationic peptides, the effect of polymyxins on the activity of phosphorylase has been examined.

MATERIALS AND METHODS

The fourth crystals of rabbit muscle phosphorylase b were prepared as in (5). AMP was removed by filtration on Sephadex G-25, and by treatment with acid-washed Norit (1 mg/mg of protein). Enzyme concentration was measured as in (6). Phosphorylase a was prepared from phosphorylase b by phosphorylation with the ammonium sulfate precipitate of the 78000Xg supernatant fraction of phosphorylase kinase (7). Phosphorylase b' was obtained from phosphorylase a by limited

proteolysis with trypsin in the presence of glucose (8).

The activity of glycogen phosphorylase was determined in the direction of glycogen synthesis under first-order kinetic conditions. The assay system contained 5 µg/ml enzyme, various concentrations of AMP, glucose-1-phosphate, and glycogen, 40 mM glycerol-2-phosphate, 30 mM 2-mercaptoethanol, 1 mM EDTA (pH 6.8 and 30°C) in a final volume of 0.2 ml. The concentrations of polymyxins used in the assay system ranged between 0.03 to 0.4 mM. Phosphate released in the enzymatic reaction was measured as in (9). When the concentration of polymyxins increases above 0.5 mM, upon the addition of the acid molybdate reagent, the solution is becoming gradually more turbid. Initial rates of phosphorylase activity were expressed in µmol of product formed per min per mg of enzyme.

Oyster glycogen (BDH) was freed of AMP as described in (10). AMP, glucose-1-phosphate, glucose-6-phosphate, and glycerol-2-phosphate were products of BDH. 2,4-diaminobutyric acid was purchased from Serva. Polymyxins were obtained from DUMEX LTD (Denmark), I.C.I.A. (Belgium), Pfizer, and Sigma.

RESULTS AND DISCUSSION

All polymyxins tested in this work, i.e., polymyxin A sulfate, polymyxin B sulfate, polymyxin D sulfate, and polymyxin E sulfate in nearly low concentrations (0.03 - 0.4 mM) were able to significantly stimulate AMP-dependent activity of phosphorylase b. This stimulation was more pronounced at suboptimal concentrations of AMP. In the absence of nucleotide, no activation of phosphorylase b can be induced by polymyxins. Fig. 1 shows that polymyxin B at 0.4 mM causes a 700% activation of the enzyme when assayed with 0.02 mM AMP, 16 mM glucose-1-phosphate, and 1% glycogen. A slight activation (15-20%) was observed even at saturating concentrations of the nucleotide (1 mM), indicating that Vmax of phosphorylase b with respect to AMP is also increased by polymyxins.

Polymyxin B is the most effective in stimulating AMP-dependent activity of phosphorylase b, while polymyxin D is the least effective (Fig. 1). This is possibly due to the lower 2,4-diaminobutyric acid content of polymyxin D as compared to polymyxin B. In fact, the position 3 of the decapeptide skeleton of polymyxins is occupied by D-Ser in polymyxin D instead of 2,4-diaminobutyric acid (Diagram 1). The sulfuric content of polymyxins (calculated as one equivalent of

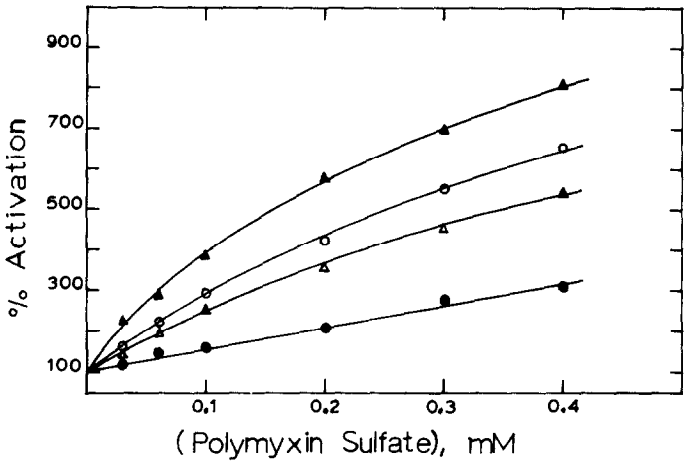


Fig. 1. Effect of polymyxins on the AMP-dependent activity of phosphorylase b. The enzyme (5 µg/ml) was assayed at pH 6.8, and 30°C with 0.02 mM AMP, 16 mM glucose-1-phosphate, 1% glycogen, and various concentrations of polymyxins mentioned in the figure. The enzyme activity without polymyxins was taken as 100%. (●) polymyxin D, (▲) polymyxin A, (○) polymyxin E, (△) polymyxin B.

sulfuric acid for each amino group of the polymyxin molecule) at the concentrations used in our experiments is too low to be able to stimulate AMP-activation of the enzyme (3). On the other hand, 2,4-diaminobutyric acid alone (1 - 100 mM) has no significant effect on the AMP-dependent activity of phosphorylase b. Also, neither saturated fatty acids (C_1 - C_{16}) nor biologically cyclic peptides such as oxytocin, vasopressin (SANDOZ LTD), and the antibiotic thiostrepton (I.C.I.A.) induce considerable activation of the AMP-phosphorylase b system. The above experiments excluded the possibility that the observed AMP-activation of phosphorylase b could have arisen from a direct contribution of either non-polycationic cyclic peptide or fatty acid residues. It appears therefore that the effectiveness of polymyxins in stimulating AMP-dependent activity of phosphorylase b is related to their polycationic peptide nature, and is possibly due to an interaction of the cationic groups of polymyxins with negatively-charged groups of the enzyme molecule. The differences in the

TABLE 1

Effect of polymyxins on the kinetic parameters for AMP binding to phosphorylase b

	K_m ($M \times 10^5$)			
Polymyxin (mM)	A	B	D	E
0	7.8 ± 0.2 (1.8 ± 0.1)			
0.03	7.1 (1.6)	6.7 (1.6)	7.4 (1.8)	6.8 (1.7)
0.06	6.3 (1.5)	6.0 (1.5)	6.8 (1.7)	5.9 (1.7)
0.10	5.6 (1.5)	5.0 (1.5)	6.2 (1.7)	5.1 (1.6)
0.20	4.3 (1.5)	2.9 (1.5)	5.3 (1.6)	3.5 (1.6)
0.30	4.0 (1.4)	2.3 (1.5)	4.5 (1.6)	2.7 (1.6)
0.40	3.0 (1.4)	2.0 (1.4)	4.4 (1.5)	2.5 (1.5)

The assays contained 5 µg/ml enzyme, 16 mM glucose-1-phosphate, 1% glycogen, and various concentrations of polymyxins. AMP concentration was varied from 0.01 to 1 mM. K_m values and Hill coefficients (given in parentheses) were determined from Hill plots (11). The mean values for K_m, and Hill coefficient for AMP in the absence of polymyxins are also quoted together with the standard deviation of the mean for five measurements.

behaviour of the polymyxins A, B, and E in activating AMP-phosphorylase b system can be ascribed to differences in their amino acid compositions.

The effect of increasing concentrations of polymyxins on the K_m value, and the Hill coefficient (n) for AMP binding to phosphorylase b is presented in Table 1. As can be seen, the enhancement of the affinity of AMP for the enzyme is accompanied by partial elimination of the allosteric interactions between the AMP binding sites of the enzyme (11). It has been reported that spermine at a concentration of 2 mM does remove the cooperative binding of AMP to phosphorylase b (2). The data presented in Table 2 show that polymyxin B at 0.3 mM in the assay system leads to (a) lowering of K_m values for the substrates glucose-1-phosphate, and glycogen, (b) increase of Vmax with respect to glucose-1-phosphate, (c) reversal of the inhibition of the enzyme caused by glucose-6-phosphate, and (d) desensi-

TABLE 2

Effect of polymyxin B on the kinetic parameters of phosphorylase b

Kinetic parameter	In the absence of polymyxin B	In the presence of 0.3 mM polymyxin B
Vmax[a] for glucose-1-phosphate	54	64.5
K_m[b] for glucose-1-phosphate (mM)	4.3	2.3
K_m[c] for glycogen (%)	0.019	0.013
Vmax[d] for AMP	50	57
K_i[e] for glucose-6-phosphate (mM)	9.1	49.5
n[e] for glucose-6-phosphate	1.9	1.1

[a]Determined at 1% glycogen, 1 mM AMP, and 2-32 mM glucose-1-phosphate from plots of 1/(velocity) versus 1/(glucose-1-phosphate) by extrapolation.

[b]Determined from Hill plots (11) from (a).

[c]Determined at 16 mM glucose-1-phosphate, 1 mM AMP, and 0.01-1% glycogen from plots of 1/(velocity) versus 1/(glycogen).

[d]Extrapolated from plots of 1/(velocity) versus 1/(AMP) from data obtained at 1% glycogen, 16 mM glucose-1-phosphate, and 0.01-1 mM AMP.

[e]Determined at 1% glycogen, 16 mM glucose-1-phosphate, 1 mM AMP, and 2.5-20 mM glucose-6-phosphate from Hill plots.

tization of allosteric interactions toward glucose-6-phosphate. Based on these results, it is suggested that the binding of polymyxins to glycogen phosphorylase b results in an enzyme form which is similar to phosphorylase a, since the latter enzyme species shows a higher affinity for AMP, no affinity for glucose-6-phosphate, and is almost devoid of allosteric interactions. The same idea has been previously suggested for polyamines, and phosphorylated tetradecapeptide (2, 12).

In contrast to phosphorylase b, AMP-phosphorylase b' system was found to be insensitive to the addition of polymyxins. Phosphorylase a is also not activated by polymyxins in the absence of AMP, while a small activation (25%) of phosphorylase a is achieved when assayed with 0.02 mM AMP (16 mM glucose-1-phosphate and 1% glycogen) in the presence of 0.3 mM polymyxin B. The crystal structure of both phos-

phorylase b (13), and phosphorylase a (14) have been solved at 3 Å resolution. The electron density for the first 19 amino acids is ill defined in phosphorylase b and it has been concluded that these residues are flexible in this enzyme form (13). However, in phosphorylase a these residues are well ordered, and the phosphate group attached to Ser-14 interacts with a basic group, probably Arg-69 (13). Our results indicate that the stimulation of AMP-phosphorylase b system by polymyxins is closely related to the flexibility of the first 19 amino acid residues. This is concluded from the inability of polymyxins to stimulate AMP-activation either of phosphorylase a where these residues are well ordered in the N-terminal region of the chain or of phosphorylase b' which lacks the first 16 amino acid residues (15).

ACKNOWLEDGEMENTS

We wish to thank Dr. Knud Andersen of DUMEX LTD, Denmark for providing us with polymyxins, and analytical data for their composition.

REFERENCES

1. Graves, D.J. and Wang, J.H. (1972) in: The Enzymes, vol. 7, pp. 435-482, Academic Press, New York, London.
2. Carty, T.J., Tu, J.-I. and Graves, D.J. (1975) J.Biol.Chem. 250, 4980-4985.
3. Sotiroudis, T.G., Oikonomakos, N.G. and Evangelopoulos, A.E. (1979) Biochem.Biophys.Res.Commun. 90, 234-239.
4. Storn, D.R., Rosenthal, K.S. and Swanson, P.E. (1977) Ann.Rev. Biochem. 46, 723-763.
5. Sotiroudis, T.G., Oikonomakos, N.G. and Evangelopoulos, A.E. (1979) Biochem.Biophys.Res.Commun. 86, 674-682.
6. Kastenschmidt, L.L., Kastenschmidt, J. and Helmreich, E. (1968) Biochemistry 7, 3590-3608.
7. Cohen, P. (1973) Eur.J.Biochem. 34, 1-14.
8. Graves, D.J., Mann, S.A.S., Philip, G. and Oliveira, R.J. (1968) J.Biol.Chem. 243, 6090-6098.
9. Fiske, C.H. and Subbarow, Y. (1925) J.Biol.Chem. 66, 375-400.
10. Helmreich, E. and Cori, C.F. (1964) Proc.Natl.Acad.Sci.U.S.A. 51, 131-138.
11. Madsen, N.B. and Schechosky, S. (1967) J.Biol.Chem. 242, 3301-3307.

12. Wang, J.H., Humniski, P.M. and Black, W.J. (1968) Biochemistry 7, 2037-2044.
13. Weber, I.T., Johnson, L.N., Wilson, K.S., Yeates, D.G.R., Wild, D.L. and Jenkins, J.A. (1978) Nature 274, 433-437.
14. Fletterick, R.J., Sygusch, J., Semple, M. and Madsen, N.B. (1976) J.Biol.Chem. 251, 6142-6146.
15. Janski, A.M. and Graves, D.J. (1979) J.Biol.Chem. 254, 4033-4039.