Vol. 86, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1979 Pages 420-427

PURIFICATION AND STRUCTURE OF A NEW NUCLEOTIDE FROM *PROTEUS MIRABILIS* THAT AMPLIFIES INDUCTION OF TYROSINE AMINOTRANSFERASE BY GLUCOCORTICOID

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Received December 18,1978

SUMMARY: A new guanosine nucleotide was purified from *Proteus mirabilis* as a factor amplifying the action of glucocorticoid. The results of Chemical analyses and acid and enzymic hydrolyses and the NMR spectra indicated that the new compound is guanosine 3'(2')-diphosphate. This compound amplifies the induction of liver tyrosine aminotransferase (EC 2.6.1.5) by dexamethasone in adrenalectomized rats, but does not itself have any glucocorticoid-like action in these animals.

Katunuma et al. demonstrated the existence of a factor in *Proteus mirabilis*, isolated from rat enteric flora, that enhances several actions of glucocorticoid, such as enzyme induction in liver, immunosuppression and the anti-inflammatory action (1). Further purification showed that this so called "Katunuma factor" consists of two components. One of these components (Factor I) has a single peak of absorption near at 260 nm and is completely inactivated by α-glucosidase (EC 3.2.1.20). The amplifying effects of partially purified Factor I on enzyme induction (2,3) and the cytotoxicity of glucocorticoid on cultured lymphoblast cells (4) have been studied. The other component (Factor II) has an absorption maximum near at 255 nm with a shoulder at about 270 nm and is inactivated by phosphodiesterase II. Factor II is

0006-291X/79/020420-08$01.00/0

present in higher concentration than Factor I, but has the same biological actions as Factor I. This paper reports the purification, chemical structure and biological action of Factor II.

MATERIALS AND METHODS

Materials. Guanosine 2'-monophosphate (2'-GMP), guanosine 3'-monophosphate (3'-GMP), guanosine 5'-monophosphate (5'-GMP), guanosine 5'-diphosphate (5'-GDP), guanosine 5'-triphosphate (5'-GTP) and guanosine 3',5'-cyclic phosphate, and 3'-nucleotidase (EC 3.1.3.6), phosphodiesterase I (EC 3.1.4.1) and phosphodiesterase II (EC 3.1.4.18) were obtained from Sigma. Polystyrene gel G3000S was purchased from Toyo Soda Co. Ltd., Japan and Dowex 1-x2 (Cl^-form) Dow Chemical Co.

Analytical procedures. Total phosphate and acid labile phosphate were determined by the method of Fiske and Subbarrow (5). Ribose was assayed by the orcinol reaction (6) and total sugar was assayed by the method of Dubois et al. (7). Guanosine concentration was determined using the equation of Cabib and Leloir (8).

Acid hydrolysis of Factor II was carried out in 1N HCl for 7, and 60 min at 100°. Nucleotides, nucleosides and bases were analyzed by thin layer chromatography on sheets of silica gel and cellulose with two different solvent systems: solvent A, 95% ethanol-1M ammonium acetate, pH 3.8, (7.5:3, by vol.); solvent B, isopropanol-ammonia-water (7:1:2, by vol.). Acid-labile phosphate was detected on cellulose plates by the method of Suzuki(9). Sugar constituents in the acid hydrolyzate of Factor II were also analyzed by thin layer chromatography with two different solvents : solvent C, phenol-0.1% ammonia (5:3, by vol.) and solvent D, butanol-pyridine-water (6:4:3, by vol.). Reducing sugars were detected on silica gel plates by the method of Stahl and Kaltenbach (10). For determination of the phosphate linkage in Factor II, it was incubated with 3'-nucleotidase (0.05 unit) in 0.2 ml of 100 mM Tris-HCl buffer, pH 7.5; with phosphodiesterase I (0.05 unit) in 0.2 ml of 100 mM Tris-HCl buffer, pH 7.5 and 25 mM $MgCl_2$; and with phosphodiesterase II (0.05 unit) in 0.2 ml of 65 mM citrate buffer, pH 6.0. After incubation at 37°, the mixtures were analyzed by thin layer chromatography and bioassay. A Varian NMR spectrometer, model HA-100 was used for proton NMR spectra. Tyrosine aminotransferase (TAT) activity, protein concentration and the biological effects of Factor II were assayed as described previously (2).

RESULTS AND DISCUSSION

Purification of Factor II. Cultivation of *P. mirabilis* and extraction of amplifying factor with perchloric acid were described previously (2). All purification procedures were carried out under ice-cold conditions. Activated charcoal (50 g/kg of cells) was added to the extract from the cells. The charcoal was washed first with 0.1N HCl and then with 10% of ethanol and the active

TABLE I

Chemical analysis of Factor II

	Ratio to acid-labile phosphate (μmoles/μmole acid-labile phosphate)
Guanosine	1.07
Acid-labile phosphate	1.00
Total phosphate	2.08
Ribose	0.96
Total sugar	0.98

compound was eluted from it with 30% of ethanol. The eluate was concentrated and applied to a Sephadex LH-20 column (2.2x62 cm). The column was eluted with butanol-pyridine-water(1:1:20, by vol.) and fractions of 3.2 ml of eluate were collected. The fractions in the two peaks of activity (tubes number 27-33 and 34-44) were each combined and concentrated. The former (tubes number 27-33) contained Factor I and the latter (tubes number 34-43) contained Factor II. The latter fraction was applied to a column of polystyrene gel G3000S (6x12 cm) equilibrated with water. The fractions of unadsorbed material were evaporated and minced with aceto nitrile at a final concentration of 70%. The resulting precipitate was dissolved in a small amount of water and subjected to paper chromatography in n-butyric acid -0.5 M ammonia (5:3, by vol.). The fraction of R_F 0.48 with ultraviolet-absorption was eluted. The eluate was passed through on a Dowex 1-x2 resin equilibrated with 0.06M NaCl in 0.03N HCl to separate Factor II from contaminant, 3'-GMP; Factor II followed 3'-GMP on the column. The fractions of Factor II were precipitated by addition of 4 volume

of acetonitril. Thin layer chromatographic estimates of the purity of Factor II showed it to be single.

Determination of Chemical Structure. The ultraviolet absorption spectra of Factor II at acidic, neutral, and alkaline pH values were identical with those of guanosine derivatives (11). The compound did not react with Elison-Morgan (12), Morgan-Elison (13) or Thiobarbituric acid (14) reagent. As shown in Table I, chemical analysis of Factor II suggests that it is a guanosine diphosphate. The results of thin layer chromatography of Factor II, its degradation products and related guanosine derivatives are summarized in Table II. Treatment of Factor II with 1N HCl for 7 min at 100° liberated predominantly 3'-GMP with a little 2'-GMP, judging from the R_F values. Treatment of Factor II with 1N HCl for 60 min at 100° liberated inorganic phosphate completely from the organic phosphate in this compound. Treatment of Factor II with phosphodiesterase II for 10 min at 37° liberated predominantly 3'-GMP. Treatment with phosphodiesterase I or 3'-nucleotidase had no detectable effect. For estimation of sugar constituents, Factor II was hydrolyzed in 1N HCl for 60 min and the hydrolyzate was analyzed by thin layer chromatography (solvent C and D). Only ribose was detected. These results suggest that Factor II is guanosine 3'(2')-diphosphate (a mixture of the 3'-and 2'-isomers).

For determination of the position of the pyrophosphate, proton NMR spectroscopy was performed and assignment of the structure is summarized in Table III. The chemical shift of H-3' of Factor II was downfield relative to guanosine and that of H-5' of Factor II was similar to that of guanosine (15). A small doublet signal at 6.02 ppm and a small multiplet signal at 5.13 ppm were also detected (data not shown) and assigned to the H-1' and H-2'

TABLE II

Thin layer chromatography of Factor II and its degradation products

	Solvent A		Solvent B	
	R_F	acid labile-p	R_F	acid labile-p
Guanine	0.02	-	0.01	-
Guanosine	0.80	-	0.60	-
2'-GMP[a]	0.27	-	0.30	-
3'-GMP[a]	0.29	-	0.35	-
5'-GMP[b]	0.32	-	0.20	-
5'-GDP[b]	0.07	+	0.10	+
5'-GTP[b]	0.03	+	0.02	+
Factor II	0.24	+	0.29	+
Factor II (hydrolysis with 1N HCl for 7 min)	0.29 (0.27)[c]	- -	0.35 (0.30)[c]	- -
Factor II (hydrolysis with 1N HCl for 60 min)	0.02	-	0.01	-
Factor II (treated with phospho-diesterase II for 10 min)	0.29 (0.24)[c]	- +	0.35 (0.29)[c]	- +
Factor II (treated with phospho-diesterase I for 60 min)	0.24	+	0.29	+
Factor II (treated with 3'-nucleotidase for 60 min)	0.24	+	0.29	+

a. Free acid of guanosine monophosphate,
b. Sodium salt of nucleotide,
c. Minor component

respectively; the chemical shift of H-2' was downfield relative to guanosine (15).

Thus from these results described above, it is concluded that Factor II is guanosine 3'(2')-diphosphate.

TABLE III

Summary of chemical shifts and coupling constants of Factor II in D_2O

Chemical shift[a], δ	Multiplicity of signal	Relative number of protons	Coupling constant, J	Assignment
ppm[b]			Hz	
3.89	broad	2	3	H-5'
4.42	multiplet	1		H-4'
4.5-4.8	multiplet	1		H-3'
4.86	multiplet	1		H-2'
5.94	doublet	1	6	H-1'
8.00	singlet	1		H-8

a. Chemical shifts at 100 MHz,
b. Shifts given in parts per million relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate

Effect of Factor II on Induction of TAT by Dexamethasone. Treatment with 19 nmoles of dexamethasone plus Factor II caused about 8-fold increase in TAT activity, whereas treatment with the same amount of dexamethasone alone resulted in 3-fold increase as shown in Table IV. Factor II alone caused no enzyme induction. Factor II was completely inactivated by phosphodiesterase II, but not by 3'-nucleotidase. Related compounds, such as 2'-GMP, 3'-GMP, 5'-GMP, 5'-GDP, 5'-GTP and guanosine 3',5'-cyclic phosphate had no effect on enzyme induction. The result of hydrolysis of Factor II with phosphodiesterase II suggests that the factor amplifying the induction is guanosine 3'-diphosphate. The dose-response profile of amplification by Factor II was also shown in Table IV. The amplification increased with increasing amounts of Factor II and a maximum of 5-to 6-fold amplification was observed over the activity with 9.5 nmoles of dexamethasone alone.

TABLE IV

Effects of Factor II and nucleotides on induction of TAT by dexamethasone

Treatment (nmoles/kg)	Specific activity (munit/mg protein)
0.9% NaCl	7.0 ± 1.26[a]
Factor II (1000)	6.4 ± 1.12
Dexamethasone (19)	23.2 ± 4.71
Dexamethasone (19) + Factor II (251)	53.5 ± 7.83
Dexamethasone (10) + Factor II (251) (hydrolysis with phosphodiesterase II)	22.6 ± 1.54
Dexamethasone (19) + Factor II (251) (hydrolysis with 3'-nucleotidase)	54.6 ± 3.97
Dexamethasone (19) + 2'-GMP (5000)	23.3 ± 2.14
Dexamethasone (19) + 3'-GMP (5000)	22.7 ± 1.42
Dexamethasone (19) + 5'-GMP (5000)	25.7 ± 1.08
Dexamethasone (19) + 5'-GDP (10000)	22.4 ± 1.12
Dexamethasone (19) + 5'-GTP (10000)	23.5 ± 1.33
Dexamethasone (19) + guanosine 3',5'-cyclic phosphate (5000)	22.2 ± 1.10
Dexamethasone (9.5)	9.0 ± 0.21
Dexamethasone (9.5) + Factor II (540)	27.8 ± 4.10
Dexamethasone (9.5) + Factor II (5400)	52.1 ± 5.03

a. Means ± S.E. of 5 adrenalectomized rats

Acknowledgments. We thank Prof. Yoshimasa Hirata for very helpful advice on NMR spectroscopy.

REFERENCES

1. Katunuma, N., Tanaka, T., Saheki, T. and Kusumi, T. (1974) Advances in Enzyme Regulation (Weber, G., ed.) Vol. 12, pp.167-183, Pergamon Press, Oxford, New York.
2. Katunuma, N., Tomino, I., Kominami, E., Tsuda, T., Kido, H. and Sanada, Y. (1977) Eur. J. Biochem. 78, 533-539.
3. Kido, H., Higashi, T. and Katunuma, N. (1977) Eur. J. Biochem. 78, 541-546.
4. Kido, H., Nakanishi, T. and Katunuma, N. (1978) Cancer Res. 38, 3100-3103.

5. Fiske, C.H. and Subbarrow, Y. (1925) J. Biol. Chem. 66, 375-400.
6. Mejbaum, W. (1939) Z. Physiol. Chem. 258, 117-120.
7. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350-356.
8. Cabib, E. and Leloir, L.F. (1954) J. Biol. Chem. 206, 779-790.
9. Suzuki, S. (1962) J. Biol. Chem. 237, 1393-1399.
10. Stahl, E. and Kaltenbach, U. (1961) J. Chromatog. 5, 351-355.
11. Beaven, G.H., Holiday, E.R. and Johnson, E.A. (1955) Nucleic acids (Chargaff, E. and Davidson, J.N., eds.) Vol. 1, pp.493-553, Academic Press, New York.
12. Blix, G. (1948) Acta Chem. Scand. 2, 467-473.
13. Reissing, J.L., Strominger, J.L. and Leloir, L.F. (1955) J. Biol. Chem. 217, 957-966.
14. Aminoff, D. (1961) Biochem. J. 81, 384-392.
15. Jones, A.J., Grant, D.M., Winkley, M.W. and Robins, R.K. (1970) J. Amer. Chem. Soc. 92, 4079-4087.