The Purification and Properties of *N*-Acetylglucosamine 6-Phosphate Deacetylase from *Escherichia coli*

By R. J. WHITE AND C. A. PASTERNAK
 Department of Biochemistry, University of Oxford

(Received 21 February 1967)

1. *N*-Acetylglucosamine 6-phosphate deacetylase and 2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase (deaminating) (EC 5.3.1.10, glucosamine 6-phosphate deaminase) of *Escherichia coli* K₁₂ have been separated by chromatography on DEAE-cellulose. 2. *N*-Acetylglucosamine 6-phosphate deacetylase has optimum pH 8.5 and *K_m* 0.8 mM. Glucosamine 6-phosphate is a product of the reaction. There appear to be no essential cofactors. Glucosamine 6-phosphate and fructose 6-phosphate inhibit deacetylation. 3. Glucosamine 6-phosphate deaminase has optimum pH 7.0 and *K_m* 9.0 mM. It is stimulated by *N*-acetylglucosamine 6-phosphate. 4. We propose that the deacetylase be termed 2-acetamido-2-deoxy-D-glucose 6-phosphate amidohydrolase (EC 3.5.1.-), with acetylglucosamine 6-phosphate deacetylase as a trivial name.

Escherichia coli K₁₂ can utilize *N*-acetylglucosamine as sole source of carbon for growth. The catabolic route is thought to involve phosphorylation to *N*-acetylglucosamine 6-phosphate, but the pathway for the degradation of this compound is not clear. The Enzyme Commission lists a mammalian enzyme (EC 5.3.1.11) (Leloir & Cardini, 1956; Roseman, 1957) that catalyses the formation of fructose 6-phosphate, acetate and ammonia. Preliminary work with bacteria (Roseman, 1959; Bates & Pasternak, 1965), on the other hand, indicates that *N*-acetylglucosamine 6-phosphate is deacetylated to glucosamine 6-phosphate, which is then degraded by a deaminase (EC 5.3.1.10) to form fructose 6-phosphate and ammonia (Scheme 1). The existence of a deacetylase yielding glucosamine 6-phosphate was established when it was shown that extracts of mutants of *E. coli* K₁₂ which lack deaminase (White, 1966) accumulate glucosamine 6-phosphate on incubation with *N*-acetylglucosamine 6-phosphate (White & Pasternak, 1965).

It seems likely that deacetylase and deaminase are separate enzymes because (White & Pasternak, 1965): (i) mutants lacking deacetylase have deaminase and vice versa; (ii) addition of glucosamine to growing cultures of *E. coli* K₁₂ causes a four- to five-fold induction of deaminase but has little effect on deacetylase (in contrast with addition of *N*-acetylglucosamine, which causes an eight- to ninefold induction of both enzymes); (iii) deacetylase and deaminase have different pH optima. In order to confirm the existence of distinct enzymes, we have now separated deacetylase and deaminase

Scheme 1. Catabolic route for *N*-acetylglucosamine in *E. coli*.

by column chromatography and report some properties of the purified enzymes.

MATERIALS

N-Acetylglucosamine and protamine sulphate (from salmon milt) were obtained from British Drug Houses Ltd., (Poole, Dorset), D-fructose 6-phosphate was from Sigma Chemical Co. (St Louis, Mo., U.S.A.), DEAE-cellulose was from Calbiochem (Los Angeles, Calif., U.S.A.) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and glucose phosphate isomerase (EC 5.3.1.9) were from C. F. Boehringer und Soehne G.m.b.h. (Mannheim, Germany). D-Glucosamine 6-phosphate (barium salt) was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and purified by adsorption on Dowex 1 (X4; Cl⁻ form) and elution with a gradient of 0.05 N-acetic acid.

N-Acetylglucosamine 6-phosphate was prepared by the

chemical acetylation of D-glucosamine 6-phosphate (Distler, Merrick & Roseman, 1958). The product was purified by adsorption on Dowex 1 (X4; acetate form) and elution with a gradient of 0.02N-HCl.

METHODS

Preparation of crude extract. *E. coli* K₁₂ 701 (250 ml.) growing exponentially in minimal medium (Davis & Mingioli, 1950) containing glucose (0.075%) was added to 16l. of the same medium (0.075% glucose) in a 20l. bottle and the culture grown with forced aeration overnight at 37°; this concentration of glucose limits growth at 1 mg. wet wt. of cells/ml. of culture. For preparation of purified enzyme (see below) *N*-acetylglucosamine (0.05%) was added and aeration continued for a further 1½ hr., to induce acetylglucosamine 6-phosphate deacetylase (White, 1966). Mutants 1-1 and 2-1, derived from *E. coli* K₁₂ 701 and lacking glucosamine 6-phosphate deaminase and acetylglucosamine 6-phosphate deacetylase respectively (White, 1966), were grown without induction by *N*-acetylglucosamine in a similar manner. The bacteria were harvested at room temperature in a Sharples Super centrifuge, washed in 200 ml. of an ice-cold solution containing 10 mM-sodium phosphate buffer, pH 6.0, containing 2-mercaptoethanol (10 mM), hereafter referred to as buffer, and resuspended in 60 ml. of buffer. All operations were conducted at 4°. The washed suspension (25 g. wet wt. of bacteria) was disrupted by passage through a French pressure cell (American Instrument Co. Inc., Silver Spring, Md., U.S.A.) and the resultant extract centrifuged at 105 000g for 1 hr. in a refrigerated Spinco centrifuge. The sediment was discarded and the supernatant retained.

Precipitation of nucleic acids. A 2% (w/v) protamine sulphate solution, pH 6.0 (40 ml.) was mixed with 80 ml. of the high-speed supernatant. After standing for 30 min. with occasional stirring, the mixture was centrifuged at 35 000g for 10 min. The sediment was discarded and the supernatant retained.

Heat treatment. The protamine sulphate supernatant (105 ml.) was heated, with constant stirring, in a 60° water bath for 6 min. and then cooled to 4°. Denatured protein was removed by centrifuging at 35 000g for 10 min. and the supernatant retained.

Ammonium sulphate fractionation. The heat-treated supernatant (92 ml.) was made up to 60% saturation with solid (NH₄)₂SO₄, allowed to stand for 30 min. and centrifuged at 35 000g for 10 min. The supernatant was discarded and the protein sediment dissolved in 10 ml. of buffer. The (NH₄)₂SO₄ was removed by dialysis against 2l. of buffer changed three times during 16 hr.

Chromatography on DEAE-cellulose. The dialysed 0-60%-saturated (NH₄)₂SO₄ fraction was applied to the top of the column (2 cm. diam. × 35 cm.) of DEAE-cellulose that had equilibrated with buffer; the protein load was kept below 1 mg. of protein/ml. of packed DEAE-cellulose. The column was washed with 100 ml. of buffer and then eluted with a gradient of 0-250 mM-NaCl in buffer (500 ml. of 0.25 M-NaCl in buffer running into a mixing vessel containing 500 ml. of buffer). A flow rate of 1 ml./min. was maintained and 9 ml. fractions were collected until the concentration of chloride (Tarnoky, 1958) in the eluate was 125 mM. Samples were assayed for glucosamine 6-phosphate

deaminase and acetylglucosamine 6-phosphate deacetylase, and *E*₂₈₀ was used as a measure of protein concentration (Warburg & Christian, 1941). Suitable fractions were pooled and solid (NH₄)₂SO₄ was added to give a 90%-saturated solution; this was allowed to stand overnight and then centrifuged at 35 000g for 10 min. Precipitated protein was dissolved in 5 ml. of buffer and the (NH₄)₂SO₄ was removed by dialysis overnight against 2l. of buffer changed three times during 16 hr. Such preparations are termed 'purified enzyme' in this paper.

Assay of enzymes. *N*-Acetylglucosamine 6-phosphate deacetylase and glucosamine 6-phosphate deaminase were assayed chemically (Levy & McAllan, 1959) or enzymically as described by Bates & Pasternak (1965), except that glucose phosphate isomerase (4 units) was added in the enzymic assays. Phosphate buffer was used instead of tris buffer (see Fig. 4) for the enzymic assay of deaminase. For the enzymic assay of deacetylase an excess of deaminase prepared from mutant 2-1 was also added. All assays were carried out at 37°. One unit of enzyme activity was expressed as 1 μmole of substrate metabolized/min.

RESULTS

Purification of acetylglucosamine 6-phosphate deacetylase. The increase in the specific activities of acetylglucosamine 6-phosphate deacetylase and glucosamine 6-phosphate deaminase during fractionation is shown in Table 1. There was little separation of the two enzymes before chromatography on DEAE-cellulose; the distribution pattern of deaminase and deacetylase in the eluate fractions is illustrated in Fig. 1. The specific activity of the pooled peaks, obtained by elution from DEAE-cellulose, indicates a 370-fold purification of deacetylase and a 420-fold purification of deaminase. Although it proved possible to obtain deaminase completely free of deacetylase, all deacetylase preparations were slightly contaminated with deaminase.

Properties of purified acetylglucosamine 6-phosphate deacetylase. The effect of pH on activity is shown in Fig. 2, the optimum activity being at about pH 8.5. The *K_m* (Lineweaver & Burk, 1934) in sodium phosphate buffer, pH 8.0, is 0.8 mM (Fig. 3). Prolonged dialysis against 10 mM-sodium phosphate buffer, pH 7.0, caused little loss of activity, indicating that no essential cofactors are involved. After prolonged incubation acetylglucosamine 6-phosphate was completely deacetylated; the reaction was not demonstrably reversible. *N*-Acetylglucosamine was not deacetylated by the purified enzyme, though crude extracts contain an extremely labile *N*-acetylglucosamine deacetylase. Table 2 illustrates the effect of several compounds on deacetylase activity; ammonia and acetate are without effect, but glucosamine 6-phosphate, and to a smaller extent fructose 6-phosphate, are inhibitors.

Identification of product. As already mentioned,

Table 1. Purification of glucosamine 6-phosphate deaminase and acetylglucosamine 6-phosphate deacetylase

Details of the fractionation procedure are given in the Methods section. Enzyme units are expressed in μ moles of substrate metabolized/min.

	Deaminase			Deacetylase		
	Total activity (units)	Sp. activity (units/mg. of protein)	Recovery (%)	Total activity (units)	Sp. activity (units/mg. of protein)	Recovery (%)
Crude cell-free extract	350	0.066	100	805	0.151	100
Protamine sulphate supernatant	98	0.084	28	204	0.174	25
Heat-treatment supernatant	98	0.143	28	170	0.241	21
0.60%-satd. (NH ₄) ₂ SO ₄ precipitate	44	0.113	15	154	0.395	19
DEAE-cellulose eluate	17	27.6	5	34	56.0	4

Fig. 1. Chromatography on DEAE-cellulose. The partially purified enzyme solution (see the Methods section) was eluted with a concentration gradient of NaCl (0–250mm) in 10mm-sodium phosphate buffer, pH 6.0. The concentrations of glucosamine 6-phosphate deaminase (●); acetylglucosamine 6-phosphate deacetylase (○), protein (—) and chloride (---) in fractions (9ml.) were measured as described in the Methods section. One arbitrary unit is equivalent to 6.5 enzyme units/mg. of protein.

Fig. 2. Effect of pH on acetylglucosamine 6-phosphate deacetylase. *N*-Acetylglucosamine 6-phosphate (0.1 μ mole) and sodium phosphate buffer (10 μ moles) (○), tris-HCl buffer (□) or glycine-NaOH buffer (●) were incubated with purified enzyme (6 μ g. of protein) in a final volume of 0.3 ml. for 5 min. at 37°. The reaction was stopped by boiling and the disappearance of acetyl-amino sugar measured chemically. One arbitrary unit is equivalent to 3.1 enzyme units/mg. of protein.

even the purest preparations of acetylglucosamine 6-phosphate deacetylase were contaminated with glucosamine 6-phosphate deaminase. This can be overcome by the use of mutant 1-1, which lacks deaminase (White, 1966), and by carrying out the reaction in tris buffer, which inhibits deaminase (Fig. 4). Table 3 shows the result of an experiment in which partially purified extracts from wild-type and mutant 1-1 were incubated with *N*-acetylglucosamine 6-phosphate. After incubation with mutant extract almost all the *N*-acetylglucosamine that has disappeared can be accounted for as

glucosamine 6-phosphate. With wild-type extract there is only a partial accumulation.

Properties of glucosamine 6-phosphate deaminase. The purified enzyme, in contrast with a crude extract of *Bacillus subtilis* (Clarke & Pasternak, 1962), is considerably more active in phosphate buffer than in tris. The pH optimum in phosphate buffer is 7.0 (Fig. 4) and the K_m (Lineweaver & Burk, 1934) at this pH is 9mm (Fig. 5). Addition of *N*-acetylglucosamine 6-phosphate causes a marked stimulation of deaminase activity (Table 4). At the substrate concentration used (1mm), *N*-acetylglucosamine 6-phosphate gave maximal stimulation (2.8-fold) at 0.22mm but caused no stimulation at all at 0.011mm.

Fig. 3. Effect of substrate concentration on acetylglucosamine 6-phosphate deacetylase. The rate was determined enzymically with 0.1 μ mole of NADP, 10 μ moles of sodium phosphate buffer, pH 8, purified glucosamine 6-phosphate deaminase (25 μ g. of protein) purified acetylglucosamine 6-phosphate deacetylase (16 μ g. of protein), glucose phosphate isomerase (4 units) and glucose 6-phosphate dehydrogenase (1.5 units) in a final volume of 0.5 ml.

Table 2. Inhibition of acetylglucosamine 6-phosphate deacetylase

Purified enzyme (0.3 μ g.) was incubated with *N*-acetylglucosamine 6-phosphate (0.1 μ mole), sodium phosphate buffer, pH 8.0 (10 μ moles), and the compounds indicated, in a total volume of 0.3 ml., for 10 min. The reaction was stopped by boiling and the disappearance of acetyl-amino sugar measured chemically.

Additions	Concn. (mm)	Activity (arbitrary units)
None	—	100*
Sodium acetate	15.0	100
Ammonium phosphate	15.0	100
Fructose 6-phosphate	3.3	82
Fructose 6-phosphate	6.6	72
Glucosamine 6-phosphate	3.3	57
Glucosamine 6-phosphate	6.6	20

* Specific activity: 27 units/mg. of protein.

DISCUSSION

Chromatography on DEAE-cellulose (Fig. 1) has shown that *N*-acetylglucosamine 6-phosphate deacetylase and glucosamine 6-phosphate deaminase are separate enzymes. The high pH optimum (Fig. 2) of deacetylase resembles that of similar enzymes from *B. subtilis* (Bates & Pasternak, 1965) and bovine parotid glands (Matushita & Takagi,

Table 3. Accumulation of glucosamine 6-phosphate during deacetylation of *N*-acetylglucosamine 6-phosphate

N-Acetylglucosamine 6-phosphate (2 μ moles), tris-HCl buffer, pH 8 (100 μ moles), and non-induced extract of wild-type or mutant 1-1 bacteria purified as far as the protamine sulphate step (12 mg. of protein for each) in a final volume of 2 ml. were incubated for 30 min. and the reaction was stopped by boiling. Amino sugar was determined chemically and enzymically, as described in the Methods section.

	Wild-type extract (μ mole)	Mutant 1-1 extract (μ mole)
<i>N</i> -Acetylglucosamine 6-phosphate disappearing (chemical method)	0.80	0.89
Glucosamine 6-phosphate appearing (chemical method)	0.51	0.82
(enzymic method)	0.44	0.78

Fig. 4. Effect of pH on glucosamine 6-phosphate deaminase. Glucosamine 6-phosphate (0.1 μ mole) and sodium phosphate buffer (10 μ moles) (O), or tris-HCl buffer (●) were incubated with purified enzyme (0.2 mg. of protein) in a final volume of 0.3 ml. for 10 min. at 37°. The reaction was stopped by heating and the disappearance of amino sugar measured chemically. One arbitrary unit is equivalent to 0.4 enzyme unit/mg. of protein.

1966). Incubation of the mutant extract with *N*-acetylglucosamine 6-phosphate (Table 3) has shown that the product of deacetylation is glucosamine 6-phosphate; although the chemical estimation of glucosamine 6-phosphate is not entirely specific, the

Fig. 5. Effect of substrate concentration on glucosamine 6-phosphate deaminase. The rate was determined enzymically, with 0.1 μ mole of NADP, 20 μ moles of sodium phosphate buffer, pH 7, purified glucosamine 6-phosphate deaminase (0.1 μ g. of protein), glucose phosphate isomerase (4 units) and glucose 6-phosphate dehydrogenase (1.5 units) in a final volume of 0.5 ml.

Table 4. Stimulation of glucosamine 6-phosphate deaminase by N-acetylglucosamine 6-phosphate

Purified glucosamine 6-phosphate deaminase was assayed as described for Fig. 5, with 1 mM-glucosamine 6-phosphate.

Concn. of acetylglucosamine 6-phosphate (mM)	Initial rate (arbitrary units)
—	1.00*
0.011	1.00
0.022	1.28
0.044	1.63
0.110	2.33
0.220	2.79
0.440	2.68

* Initial rate: 26 units/mg. of protein.

enzymic estimation is (Comb & Roseman, 1962). The products of deacetylation for the mammalian enzyme (Matshita & Takagi, 1966) have also been identified as glucosamine 6-phosphate and acetate. We therefore propose that the enzyme be designated 2-acetamido-2-deoxy-D-glucose 6-phosphate amidohydrolase (EC 3.5.1.-), with acetylglucosamine 6-phosphate deacetylase as a trivial name. Little is known about the specificity of the enzyme from *E. coli*, but Bates & Pasternak (1965) have shown that extracts of *B. subtilis* deacylate formyl-, acetyl-, propionyl- and *n*-butyryl-glucosamine 6-phosphate. The large variety of *N*-acyl glucosaminides that can act as growth factors for *Lactobacillus bifidus* var.

pennsylvanicus (Lambert & Zilliken, 1965) may also reflect a similar lack of specificity of deacylation. It has been shown that deacylation occurs before incorporation of amino sugars into bacterial cell walls (Bates & Pasternak, 1965; Lambert, Saito & Veerkamp, 1965; White, 1966).

A previous purification of glucosamine 6-phosphate deaminase from *E. coli* (Comb & Roseman, 1958) gave a 120-fold increase in activity; the present method results in a 420-fold purification. Comb & Roseman (1958) report K_M 7.1 mM in tris-hydrochloric acid buffer at pH 7.8; we find one of 9.0 mM in phosphate buffer at pH 7.0, the buffer having a marked effect on activity (Fig. 4). The reason for the stimulation of deaminase by *N*-acetylglucosamine 6-phosphate (Table 4), previously noted by Comb & Roseman (1958), is obscure. It could be important as a means of regulating the pool size of glucosamine 6-phosphate during growth on *N*-acetylglucosamine; work with mutants of *E. coli* (White, 1966) has suggested that the concentration of glucosamine 6-phosphate plays a key role in the control of amino sugar metabolism. It is noteworthy that a similar stimulation of deaminase has been described for hog kidney (Comb & Roseman, 1958), *B. subtilis* (Bates & Pasternak, 1965) and neoplastic mast cells (P815) in culture (C. A. Pasternak, unpublished work).

We are indebted to the Medical Research Council for the award of a scholarship (to R. J. W.).

REFERENCES

- Bates, C. J. & Pasternak, C. A. (1965). *Biochem. J.* **96**, 147.
- Clarke, J. S. & Pasternak, C. A. (1962). *Biochem. J.* **84**, 185.
- Comb, D. G. & Roseman, S. (1958). *J. biol. Chem.* **232**, 807.
- Comb, D. G. & Roseman, S. (1962). In *Methods in Enzymology*, vol. 5, p. 422. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Davis, B. D. & Mingioli, E. S. (1950) *J. Bact.* **60**, 17.
- Distler, J. J., Merrick, J. M. & Roseman, S. (1958). *J. biol. Chem.* **230**, 497.
- Lambert, R., Saito, Y. & Veerkamp, J. H. (1965). *Arch. Biochem. Biophys.* **110**, 341.
- Lambert, R. & Zilliken, F. (1965). *Arch. Biochem. Biophys.* **110**, 544.
- Leloir, L. F. & Cardini, C. E. (1956). *Biochim. biophys. Acta*, **20**, 33.
- Levy, G. A. & McAllan, A. (1959). *Biochem. J.* **73**, 127.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Matshita, Y. & Takagi, Y. (1966). *Biochim. biophys. Acta* **124**, 204.
- Roseman, S. (1957). *Fed. Proc.* **15**, 340.
- Roseman, S. (1959). *Annu. Rev. Biochem.* **28**, 545.
- Tarnoky, A. L. (1958). *Clinical Biochemical Methods*, p. 50. London: Hilger and Watts Ltd.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
- White, R. J. (1966). D.Phil. Thesis: University of Oxford.
- White, R. J. & Pasternak, C. A. (1965). *Biochem. J.* **97**, 12 p.