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# BIOSYNTHESIS OF RESPIRATORY TRACT MUCINS

## II. CONTROL OF HEXOSAMINE METABOLISM BY L-GLUTAMINE:D-FRUCTOSE 6-PHOSPHATE AMINOTRANSFERASE*

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SUMMARY

1. L-Glutamine:D-fructose 6-phosphate aminotransferase (EC 2.6.1.16) was isolated and partially purified from bovine trachea. The enzyme exhibited a broad pH optima from 6.5 to 7.5.

2. The allosteric inhibition of the enzyme by UDP-*N*-acetylglucosamine was investigated in detail. A Hill plot of the kinetic data indicated negative cooperativity in the presence of UDP-*N*-acetylglucosamine.

3. An analogue of L-glutamine, 6-diazo-5-oxo-L-norleucine exhibited competitive inhibition with respect to L-glutamine.

4. A discussion of these findings with reference to the regulation of hexosamine biosynthesis in the mucus-producing structures of the trachea is presented.

## INTRODUCTION

The mucous secretion produced by the mammalian respiratory tract contains glycoproteins. This secretion maintains the normal function of the respiratory tract and is an important factor in obstructive lung diseases[1]. *N*-Acetylhexosamines are major constituents of the oligosaccharide side chains of tracheobronchial mucous glycoproteins[2]. The key enzyme responsible for the synthesis of these hexosamines is believed to be L-glutamine:D-fructose 6-phosphate aminotransferase[3–5] (EC 2.6.1.16). This enzyme catalyzes the transfer of the amide nitrogen from glutamine to fructose 6-phosphate to give glucosamine 6-phosphate, the immediate precursor of other aminosugars. Feedback inhibition of the enzyme by UDP-*N*-acetylglucosamine (UDP-GlcNAc) was first demonstrated in rat liver by Kornfeld *et al.*[6]. The apparent wide distribution of the enzyme[5] suggests the aminotransferase is an important site of control in glycoprotein biosynthesis[8–10].

Abbreviation: UDP-GlcNAc, UDP-*N*-acetylglucosamine.

* Paper I in this series is ref. 13.

L-Glutamine:D-fructose 6-phosphate aminotransferase isolated from various sources exhibits different feedback inhibition patterns[8-12]. Mazlen *et al.*[11,12] noted that UDP-GlcNAc converts the retinal enzyme to a form which is inhibitable by one of its substrates, glutamine. Preliminary experiments have indicated that the enzyme from bovine trachea is susceptible to glutamine inhibition which is independent of feedback control[9].

This paper describes some of the properties of the bovine respiratory tract enzyme including feedback inhibition by UDP-GlcNAc and is part of a detailed study of the biosynthesis and secretion of respiratory tract mucins[13].

## MATERIALS AND METHODS

### *Chemicals*

Glutamine, glucose 6-phosphate and glucosamine 6-phosphate were purchased from Sigma Chemical Co., UDP-GlcNAc and fructose 6-phosphate from Boehringer Mannheim Corp., and $(NH_4)_2SO_4$ (ultrapure) was obtained from Mann. The 6-diazo-5-oxo-L-norleucine was prepared in our laboratory. All other chemicals were of the highest quality available from commercial sources and were used without further purification.

### *Preparation of tracheal enzyme*

All operations were conducted at 4 °C. The mucosal linings of fresh bovine tracheas, obtained from a local slaughter house and kept on ice until used, were gently removed by scraping with a scalpel. The scrapings were homogenized in a Waring blender using 2 vol. of 0.154 M KCl–1 mM EDTA–12 mM glucose 6-phosphate (pH 7.5). The homogenate was centrifuged at 105 000 × *g* for 1 h. The supernatant was brought to 40% saturation by adding a saturated $(NH_4)_2SO_4$ solution (pH 7.0) and the mixture centrifuged at 18 000 × *g* for 10 min. The aminotransferase was then precipitated by bringing the supernatant to 55% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 2 ml of 0.05 M phosphate–1 mM EDTA (pH 7.5) and passed through a 10-ml Sephadex G-25 column previously equilibrated with the same buffer to remove $(NH_4)_2SO_4$. After elution with fresh buffer, the fractions containing protein were pooled and used for enzyme assay. The enzyme prepared in this way routinely contained 0.005–0.01 unit of enzyme per mg protein. Units of enzyme activity are defined as μmoles of glucosamine 6-phosphate formed per min. Aged enzyme preparations were stored at −15 °C as packed $(NH_4)_2SO_4$ precipitates for 2 months.

### *Assay of aminotransferase activity*

Routine incubation mixtures contained the following components in a final volume of 1 ml: 10 μmoles fructose 6-phosphate, 40 μmoles sodium phosphate buffer (pH 7.4), 1 μmole disodium EDTA, and, unless otherwise indicated, 2 μmoles glutamine. Incubations, started by addition of enzyme, were for 30 min at 37 °C. The reaction was terminated by boiling for 2 min. The denatured protein was removed by centrifugation and an aliquot of the supernatant was used for hexosamine determination. All values were corrected by a zero-time control or by controls incubated either

without enzyme or without substrates. Recoveries of added glucosamine 6-phosphate were greater than 95%.

*Analytical methods*

Glucosamine 6-phosphate was assayed by a modification of the Levvy and McAllan method[14]. To a 0.3-ml sample was added 0.08 ml of 1.5% acetic anhydride in acetone (ice-cold freshly prepared) and 0.2 ml of 0.8 M potassium borate, pH 9.1. The tubes were capped, boiled for 30 min and then cooled on ice; 2.4 ml of a dimethylaminobenzaldehyde reagent[15] were added and the mixtures incubated at 37° C for 20 min. The extinction was measured at 585 nm with the absorbance being linear to 0.2 μmole. Internal standards of glucosamine 6-phosphate were processed in control experiments to correct for interference by other compounds in the Morgan–Elson reaction. Protein was determined by the method of Lowry *et al.*[16] using crystalline bovine serum albumin as a standard. Kinetic parameters were determined from computer-generated linear regression analysis of the data by the method of Wilkinson[17].

*Determination of UDP-N-acetylhexosamine concentration in tracheal mucosa*

The level of nucleotide hexosamine in tracheal mucosa was determined on perchloric acid extracts of the tissue. Mucosal scrapings were extracted in 0.5 M perchloric acid at 4 °C. The homogenate was centrifuged for 15 min at 10 000 × *g* and the supernatant solution was neutralized with KOH. Following removal of the potassium perchlorate by centrifugation, an aliquot of the supernatant was passed through a 0.7 cm × 3 cm Ag 1-X2 (100–200 mesh) chloride column. The column was washed with 10 ml of water and the nucleotide sugars eluted with 5 ml HCl[18]. The HCl eluates were heated for 10 min at 100 °C, to hydrolyze the aminosugars esterified at position 1, and then neutralized by shaking with AG 1-X2 bicarbonate resin (20–50 mesh). The resin was removed by centrifugation and an aliquot of the supernatant was used for determination of hexosamine content. The values were corrected for 80% recovery when an internal standard of UDP-GlcNAc was carried through the procedure.

## RESULTS

*Enzyme assays*

Because of the relative instability of the enzyme, all buffers used in its preparation contained 12 mM glucose 6-phosphate as a protective agent[7,10]. During initial purification studies, attempts were made to further purify the 40%–55% saturated $(NH_4)_2SO_4$ preparation on DEAE-cellulose as originally recommended by Ghosh *et al.*[5]. However, enzyme preparations eluted from the DEAE-cellulose, while catalytically of greater specific activity than the $(NH_4)_2SO_4$ preparations, had lost their capacity to be inhibited by UDP-GlcNAc. A similar observation was reported by Winterburn and Phelps[19,20] during purification of the liver enzyme.

Since we are interested in the regulatory activity of the enzyme, we used the partially purified $(NH_4)_2SO_4$ fraction in the experiments reported below. This preparation of the enzyme is presumably similar to that present *in vivo*[7], rather than a form that has become desensitized to the action of its modifier.

All kinetic studies were made under conditions where glucosamine 6-phosphate formation increased linearly as a function of time, and enzyme activity was proportional to concentration of enzyme. Due to the instability of the enzyme the specific activity varied from one experiment to another.

*Properties of tracheal mucosal aminotransferase*

Cell fractionation studies indicated that enzyme activity was localized in the cell supernatant. As reported[9] the purified enzyme exhibited a $K_m$ of 0.2 mM for glutamine with substantial substrate inhibition occurring at higher concentrations. Aged enzyme preparations showed similar substrate inhibition patterns. Determination of the $K_m$ for fructose 6-phosphate was imprecise since the enzyme preparations still retained glucose phosphate isomerase (EC 5.3.1.9). As a corollary to the previously determined kinetic constants[9], the data were also plotted according to the empirical Hill equation[21]. Within the range of substrate concentrations covered the value of $\bar{n}$ in the Hill plot was unity, signifying no homotropic cooperative effects between substrate binding sites.

*Effect of pH on enzyme activity*

The pH dependence of the enzyme was studied using a series of phosphate–EDTA buffers covering the pH range 6.0–8.5. The aminotransferase showed a broad pH optimum from 6.5 to 7.5 (Fig. 1).

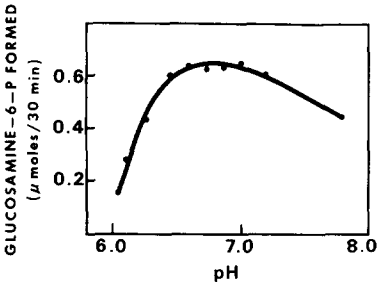
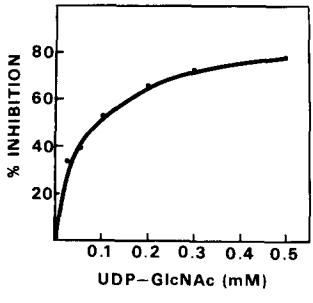



Fig. 1. Activity of enzyme as a function of pH. Incubation mixtures, prepared and assayed as described under Materials and Methods, were incubated at 37 °C for 30 min.

*Inhibitory effects of UDP-GlcNAc*

UDP-GlcNAc was a potent inhibitor of the enzyme. The inhibition was hyperbolic in shape but usually reached only 75–80% inhibition, even at very high concentrations of UDP-GlcNAc (Fig. 2). As reported[9], increasing substrate levels of glutamine caused a reduction in enzyme activity; however, the curves for percentage inhibition *versus* UDP-GlcNAc concentration were almost superimposable for different glutamine concentrations. A Dixon plot[22] at two concentrations of fructose 6-phosphate (Fig. 3) gave a $K_i$ for UDP-GlcNAc of 0.04 mM. When aged enzyme preparations were tested there was no susceptibility to inhibition by UDP-GlcNAc at levels up to 0.2 mM.

For an enzyme possessing ligand interacting sites it has been shown by Atkinson *et al.*[23], that the Michaelis equation may be put in the form



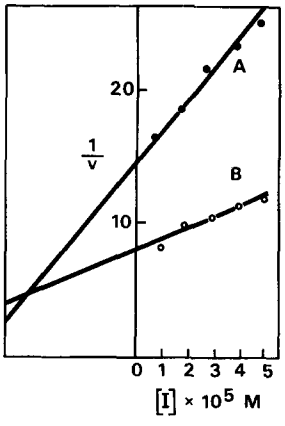




Fig. 2. Effect of UDP-GlcNAc concentration on reaction rate. The enzyme was assayed under the standard conditions except that UDP-GlcNAc was added at the concentrations indicated.

Fig. 3. Dixon plot of inhibition by UDP-GlcNAc at two concentrations of fructose 6-phosphate. $v$ is expressed as $\mu$moles glucosamine 6-phosphate formed in 30 min. A, 5 mM and B, 10 mM fructose 6-phosphate, respectively. The slopes and intercepts were determined by computer-generated linear regression analysis on five points for each line. Correlation coefficients for the lines were in each case greater than 0.98.

$$\log\left(\frac{v}{V-v}\right) = \bar{n}\log[S] - \log K \qquad (1)$$

Also known as the Hill equation[21], it has been used widely in analyzing allosteric interactions of regulatory enzymes[23,25], where the value of the slope ($\bar{n}$) is considered to be a measure of the number of interacting sites as well as the strength of their interactions. For an enzyme subject to the allosteric effects of an inhibitor it has been shown by Levitzki and Koshland[25] that the Hill equation may be transformed to

$$\log\left(\frac{v}{v_i} - 1\right) = \log\frac{1}{K_i} + \bar{n}\log[I] \qquad (2)$$

where $v$ is the velocity of reaction in the absence of inhibitor, and $v_i$ is the velocity in the presence of the inhibitor.

The effect of UDP-GlcNAc on the activity of the aminotransferase has been

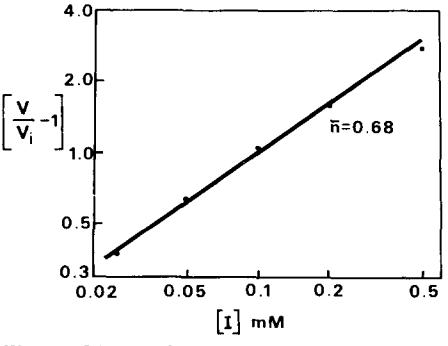



Fig. 4. Hill plot for UDP-GlcNAc inhibition plotted according to the equation: $\log(v/v_i - 1) = \log 1/K_i + \bar{n}\log[I]$ where $v$ is enzyme activity in the absence of inhibitor and $v_i$ is activity in the presence of a given concentration of UDP-GlcNAc.

plotted in this manner and is shown in Fig. 4. This plot of $\log(v/v_i - 1)$ against log [UDP-GlcNAc concentration] gave a straight line with slope less than 1, indicating possible negative cooperative effects. The figure shows that the cooperativity index[24] is large and a far higher concentration of UDP-GlcNAc than is feasible would be required to produce complete inhibition.

*Effect of diazonorleucine*

In common with other glutamine dependent transaminases, inhibition of bacterial and rat liver glucosamine 6-phosphate synthetase by 6-diazo-5-oxo-L-norleucine, an analogue of L-glutamine, has been reported[5,25]. The tracheal enzyme was similarly

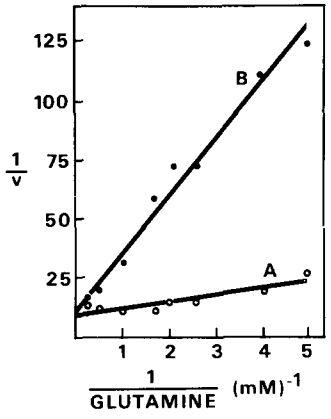



Fig. 5. Effect of 6-diazo-5-oxo-L-norleucine on $K_m$ for glutamine. Reaction mixtures were as described in the text, with the following concentrations of 6-diazo-5-oxo-L-norleucine, A, none; B, 0.01 mM. Slopes were determined from a computer-generated linear regression of eight points for each line. Correlation coefficients for the lines were greater than 0.98.

strongly inhibited (Fig. 5). The kinetics of 6-diazo-5-oxo-L-norleucine inhibition were competitive with respect to glutamine and the $K_i$ calculated from the slopes in Fig. 5 was $1 \cdot 10^{-6}$ M.

*Molecular weight of enzyme*

This was determined in two ways, by the technique of gel filtration using a calibrated Sephadex G-200 column prepared as described by Andrews[27], and by sedimentation on a sucrose gradient[28] using enzymes of known molecular weight as standards. Both methods indicated a molecular weight of approximately 350 000 for the active enzyme fraction with the assumption that the shape of the aminotransferase was approximately spherical.

*Level of UDP-GlcNAc in tracheal epithelial tissue*

Determinations of the intracellular UDP-GlcNAc concentration in tracheal linings gave values ranging from 0.10 to 0.16 mM assuming that the intracellular water for tracheal epithelial scrapings is 80% of the wet weight.

## DISCUSSION

In the biosynthesis of the various sugar nucleotides, considerable evidence suggests the presence of feedback mechanisms which determine the concentration of sugar nucleotides in the cell[6]. These compounds are important intermediates in mucin biosynthesis, acting as donors of the monosaccharide residues of the constituent glycoproteins. Our studies show tracheal aminotransferase has properties characteristic of enzymes subject to feedback inhibition, with UDP-GlcNAc acting as a non-competitive inhibitor with respect to glutamine. The enzyme is also subject to glutamine inhibition which is independent of the feedback mechanism[9]. With liver[7,29] and retinal enzymes[11] substrate inhibition by glutamine is induced in the presence of UDP-GlcNAc. The induction of negative cooperativity by UDP-GlcNAc seen in the present experiments could explain the failure to obtain complete inhibition with the feedback inhibitor[10,12,19].

Kornfeld[10] suggested the liver enzyme has a separate allosteric binding site for UDP-GlcNAc distinct from the active site. This was confirmed by Winterburn and Phelps[19,20], on the basis of thermal and chemical desensitization of the UDP-GlcNAc inhibition. The observation that prolonged contact between the enzyme and DEAE-cellulose resulted in a preparation that, although catalytically active, is devoid of inhibition by UDP-GlcNAc indicates that in the case of the tracheal enzyme the feedback inhibitor is probably bound at a site separate from the catalytic one.

The aminotransferase isolated from bovine tracheal tissue has a molecular weight of approximately 350 000. This agrees closely with values of 340 000–400 000 reported for the liver enzyme[10,19]. When this value is compared to a molecular weight of approximately 100 000 for the bacterial enzyme[10], it suggests that the mammalian enzyme may be comprised of subunits. It is tempting to speculate that, in the case of the tracheal aminotransferase, these active subunits would each have a site where glutamine could bind causing substrate inhibition[9], as well as the active site where glucosamine 6-phosphate is formed. However, only the polymeric form of the enzyme, not the active subunits would be susceptible to UDP-GlcNAc inhibition. In its native form the enzyme would exist almost entirely as a polymer, but on isolation and aging, would break down into active subunits. Thus, the degree of inhibition of UDP-GlcNAc may be an indication of the amount of enzyme in the native polymeric form.

If one assumes the tracheal intracellular concentration of UDP-GlcNAc (0.10–0.16 mM) is evenly distributed, then the aminotransferase should be combined *in vivo* with the feedback inhibitor and the full potential activity of the enzyme would not be realized. Normal activity of the aminotransferase would be that displayed by a heavily inhibited enzyme. The protection by UDP-GlcNAc of the liver enzyme from irreversible alkylation by Duazomycin A observed *in vivo*[7] supports the concept of an enzyme combined with its inhibitor. The availability of this latent activity would permit rapid synthesis of glucosamine 6-phosphate upon demand and without *de novo* biosynthesis of enzyme.

Tracheal aminotransferase is located in the soluble fraction of the cell, as are the remainder of the enzymes responsible for UDP-GlcNAc formation from fructose 6-phosphate. Consequently, the enzyme is at a central location in carbohydrate metabolism and will compete for fructose 6-phosphate with the other pathways utilizing

hexosemonophosphates. Thus, the primary control on the flow of metabolites through the hexosamine pathways, is at the level of the aminotransferase.

Control of the synthesis of glucosamine 6-phosphate and UDP-GlcNAc may play an important role in the secretion of mucins by tracheal epithelial goblet and mucosal gland cells. Modulation of the activities of the enzymes involved in the metabolism of these compounds is essential since any accumulation of UDP-GlcNAc will automatically prevent its own synthesis. When the rate of synthesis and secretion of mucus is altered the cell is able to rapidly adjust the rate of UDP-GlcNAc synthesis to its requirement for this compound. The level of the aminotransferase may thus be kept high enough to cope with increased demand without imposing on the cell the continuous overproduction of UDP-GlcNAc. Similar controls of the synthesis of nucleotide sugars appear to regulate the synthesis of cell wall materials[30].

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