The Binding of Substrates and Modifiers to Glucosamine Synthetase

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1. The binding of substrates and effectors to glucosamine synthetase (L-glutamine-D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) was studied by using the ligand to alter the denaturation rate of the enzyme. The free enzyme bound fructose 6-phosphate, glucose 6-phosphate and UDP-*N*-acetylglucosamine, but not glutamine, AMP or UTP. Glucose 6-phosphate and AMP increased the binding of UDP-*N*-acetylglucosamine whereas UTP decreased the interaction between the enzyme and the feedback inhibitor. UDP-*N*-acetylglucosamine induced a glutamine-binding site on the enzyme. 2. Selective thermal or chemical denaturation revealed that the UDP-*N*-acetylglucosamine-binding site was not located at the catalytic site. The UTP site could not be distinguished from that for the nucleotide sugar. The AMP- and glucose 6-phosphate-binding sites were distinct from the catalytic and feedback-inhibitor-binding sites. 3. The specificity of the glutamine-binding site was investigated by using a series of potential analogues. 4. A model is proposed for the action of the effectors and the mechanism of the reaction discussed in kinetic and chemical terms.

The purification, steady-state properties and regulation of glucosamine synthetase (L-glutamine-D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) are described in the preceding papers (Winterburn & Phelps, 1971*a,b*). In the latter paper it was demonstrated that AMP and glucose 6-phosphate increased the apparent binding of the feedback inhibitor, UDP-*N*-acetylglucosamine, on the enzyme, whereas UTP behaved as a competitor for the inhibitor, so leading to activation. Since these three modifiers of the inhibition by UDP-*N*-acetylglucosamine only operated in the presence of the feedback inhibitor, the question was raised as to how these effects were exerted. It is the object of the present paper to enquire into this problem and in particular to present evidence on the number and the interrelationships of the various binding sites for substrates and ligands. Finally a plausible model for the mechanism of action of this complex regulatory enzyme is described.

MATERIALS AND METHODS

L-Asparagine and L-lysine were purchased from Mann Research Laboratories Inc., New York, U.S.A. L- α -Amino- β -ureidopropionic acid (albizzin) and DL-5-hydroxylysine hydrochloride were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

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S-Carbamoyl-L-cysteine was a product of Fluka A.-G. Buchs, Switzerland. L- α -Amino-*n*-butyric acid, γ -methylglutamate, γ -ethyl glutamate, L-methionine-D,L-sulphoxide and L-norleucine were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. *O*-Diazocetyl-L-serine (azaserine) was a gift from Parke, Davis and Co. Ltd., Hounslow, Middx., U.K. *N*-Acetyl-6-diazo-5-oxo-L-norleucine (Duazomycin A) was a gift supplied by The John L. Smith Memorial for Cancer Research, Chas. Pfizer and Co. Inc., Maywood, N.J., U.S.A., where the material was produced under the support of National Institutes of Health Contract PH43-68-45 with the National Cancer Institute. All other materials were as described by Winterburn & Phelps (1971*a,b*).

Glucosamine synthetase was prepared from rat liver in either its unstabilized (preparation I) or stabilized (preparation II) forms as described by Winterburn & Phelps (1971*a*). The enzyme as eluted from the second DEAE-cellulose column (stage 4) was used for these studies.

The assay conditions for the measurement of glucosamine synthetase activity and the determination of the control ratio were as defined by Winterburn & Phelps (1971*a*). The sensitivity of the enzyme to AMP, glucose 6-phosphate or UTP was expressed in terms of a modifier ratio. The velocity was measured in the standard assay with both substrates saturating in the presence of the modifier and UDP-*N*-acetylglucosamine (final concn. 0.1 mM). The ratio of this velocity to that with the modifier omitted was termed the modifier ratio. The concentrations of AMP, glucose 6-phosphate and UTP employed in the incubation mixture were 2.0, 1.5 and 2.5 mM respectively.

RESULTS

Experimental approach to the investigation of ligand binding. The instability of glucosamine synthetase to dialysis and gel filtration in the absence of suitable protectors vitiated the use of these techniques in investigating ligand binding. This problem was overcome by using the ligand under investigation to alter the inactivation rate of the enzyme under controlled conditions. This effect had been employed previously to infer substrate binding and has been discussed by Frieden (1963); a similar technique has been described by O'Sullivan & Cohn (1966) and Avramovic & Madsen (1968).

The stability of glucosamine synthetase (preparation I) was investigated at 0°C, 10°C and 15°C and pH 7.5 by removing samples at intervals and assaying for total activity (Fig. 1). The inactivation of the enzyme was biphasic with the initial phase being completed within 5 h of preparation of the sample. All subsequent stabilization experiments were performed at 10°C in 50 mM-tris-10 mM-cacodylic acid-5 mM-EDTA-5 mM-GSH-100 mM-KCl, pH 7.5, on the second phase of the inactivation, during which period the reaction was of an apparent first-order.

Fig. 1. Influence of temperature on the stability of glucosamine synthetase (stage 4 of preparation I) in 50 mM-tris-10 mM-cacodylic acid-5 mM-EDTA-5 mM-GSH-100 mM-KCl, pH 7.5. The protein concentration was 1.3 mg/ml. Samples were removed at intervals and assayed for total remaining activity as described in the text, this being expressed as a percentage of the initial activity. The temperatures were: ○, 0°C; △, 10°C; □, 15°C.

The binding of a ligand, X, was measured by calculating the change induced in the rate constant for this first-order denaturation. The model chosen, essentially according to the procedure of London, McHugh & Hudson (1958), was the simplest possible and served the purpose of describing rather than accurately quantifying a series of observations.

The model:

gave the equation for the dissociation constant, K_x , for EX:

$$K_x = \frac{[X]k_x}{k - k_x}$$

where k is the rate constant for the apparent first-order denaturation and k_x is the rate constant in the presence of the ligand. It should be noted that this relationship does not hold for the contingency that the rate constant for the denaturation of EX is equal to that of E; this situation is indistinguishable from the case where X has not bound, i.e. $K_x = \infty$.

Investigation into binding of substrates. Glutamine had negligible effect on the first-order rate constant even at high concentrations (6 mM only evoked an 8% decrease). It was concluded that glutamine did not bind to the free enzyme.

The action of the other substrate, fructose 6-phosphate, was complex. The initial phase was an apparent reactivation of partially denatured enzyme, followed by an inactivation, the magnitude of both effects being concentration-dependent. This reactivation effect, shown in Fig. 2(a), was similar to one reported by Wakid & Mansour (1965) for guinea-pig heart phosphofructokinase. Although the higher fructose 6-phosphate concentrations stabilized, as shown in Fig. 2(b), low concentrations (less than 0.05 mM) increased the apparent rate constant for the denaturation. Behaviour such as this can be interpreted in terms of several binding sites of differing affinities for the same ligand, each binding event severally influencing the rate constant for the denaturation process (e.g. see Frieden, 1963). The interpretation of these results is that the free enzyme is capable of binding fructose 6-phosphate, and that there exists probably more than one binding site for this ligand.

Binding of modifiers. The enzyme was stabilized by adding 0.1 mM-UDP-N-acetylglucosamine. This was reflected in a decrease of the rate constant from the unstabilized value of $6.2 \times 10^{-2} \text{ h}^{-1}$ to $1.6 \times 10^{-2} \text{ h}^{-1}$ in the presence of the nucleotide sugar (Fig. 3). This gave an apparent K_i for UDP-N-acetylglucosamine of $3.5 \times 10^{-5} \text{ M}$.

Fig. 2. (a) Effect of fructose 6-phosphate on the second phase of the spontaneous denaturation at 10°C and pH 7.5 (see Fig. 1). The protein concentration was 1.9 mg/ml. After 2.5 h fructose 6-phosphate was added to each sample at constant dilution (5%, v/v) to yield the following final concentrations: O, control (water added); Δ, 0.01 mM; □, 0.025 mM; ●, 0.05 mM; ▲, 0.10 mM; ■, 1.0 mM. (b) Dependence of the apparent rate constant, k , for the denaturation phase shown in (a) on the fructose 6-phosphate concentration.

Fig. 3. Influence of UTP and UDP-*N*-acetylglucosamine on the rate of denaturation at 10°C. The conditions were as detailed for Fig. 2(a) except that the protein concentration was 1.3 mg/ml. O, Control; Δ, 0.1 mM-UDP-*N*-acetylglucosamine; □, 2 mM-UTP + 0.1 mM-UDP-*N*-acetylglucosamine.

UTP (2 mM) increased the rate constant by only 4%, corresponding to a dissociation constant of 5×10^{-2} M. Addition of the same concentration of UTP in the presence of 0.1 mM-UDP-*N*-acetylglucosamine decreased the protective action of the nucleotide sugar (Fig. 3). The rate constant was raised threefold to $4.6 \times 10^{-2} \text{ h}^{-1}$, corresponding to

an increase in the K_i for UDP-*N*-acetylglucosamine from 3.5×10^{-5} M to 2.9×10^{-4} M.

AMP (2 mM) by itself had no detectable effect on the rate constant. However, the addition of 1 mM-AMP lowered the rate constant in the presence of 0.1 mM-UDP-*N*-acetylglucosamine from $1.6 \times 10^{-2} \text{ h}^{-1}$ to $5.3 \times 10^{-3} \text{ h}^{-1}$, corresponding to a decrease in the apparent K_i for the nucleotide sugar to 9.5×10^{-6} M.

In the context of glucosamine synthetase, glucose 6-phosphate has been variously quoted as being a stabilizer (Pogell & Gryder, 1957), having a slight effect (Danishefsky & Deutsch, 1968) or having no influence (Ghosh, Blumenthal, Davidson & Roseman, 1960). In the present studies this compound had a pronounced protective capability: 1 mM-glucose 6-phosphate lowered the rate constant from $1.04 \times 10^{-1} \text{ h}^{-1}$ to $3.2 \times 10^{-2} \text{ h}^{-1}$. This corresponded to a dissociation constant for glucose 6-phosphate of 4.4×10^{-4} M. Gryder & Pogell (1960) observed that galactose 6-phosphate also exerted a stabilizing effect on the enzyme; however, at 1 mM neither it nor mannose 6-phosphate affected the rate constant. The addition of 1 mM-glucose 6-phosphate enhanced the protective capacity of UDP-*N*-acetylglucosamine. The rate constant in the presence of UDP-*N*-acetylglucosamine ($1.6 \times 10^{-2} \text{ h}^{-1}$) was lowered by the addition of 1 mM-glucose 6-phosphate to $5.5 \times 10^{-3} \text{ h}^{-1}$, which corresponded to a glucose 6-phosphate dissociation constant of 5.2×10^{-4} M. The similarity of this value to that obtained in the absence of UDP-*N*-acetylglucosamine suggested that the binding of the feedback inhibitor did not

Fig. 4. Influence of glutamine on the denaturation rate at 10°C in the presence and absence of UDP-*N*-acetylglucosamine. The conditions were as detailed for Fig. 2(a) except that the protein concentration was 1.7 mg/ml. ○, Control; □, 6 mM-glutamine; ●, 0.1 mM-UDP-*N*-acetylglucosamine; ■, 0.1 mM-UDP-*N*-acetylglucosamine + 6 mM-glutamine.

Fig. 5. Temperature-dependence of the first-order denaturation rate constant, k (min^{-1}), in the range 42–51°C. The enzyme preparation (preparation II, 1.1 mg/ml) was in the buffer detailed in Fig. 1 to which was added 1 mM-fructose 6-phosphate.

influence the binding of glucose 6-phosphate. However, the binding of the glucose 6-phosphate did lower the apparent K_i for UDP-*N*-acetylglucosamine from $3.5 \times 10^{-5} \text{ M}$ to $2.1 \times 10^{-5} \text{ M}$.

The substrate inhibition by glutamine observed in the presence of UDP-*N*-acetylglucosamine (Winterburn & Phelps, 1971b) suggested that the feedback inhibitor induced a second, stronger, binding site for this amino acid. Whereas on its own 6 mM-glutamine only decreased the rate constant by 8%, corresponding to a dissociation constant of $7.5 \times 10^{-2} \text{ M}$, with UDP-*N*-acetylglucosamine (0.1 mM) present the same change in glutamine concentration lowered the rate constant from $2.7 \times 10^{-2} \text{ h}^{-1}$ to $1.0 \times 10^{-2} \text{ h}^{-1}$ (Fig. 4). This gave a dissociation constant of $3.5 \times 10^{-3} \text{ M}$. It was evident that UDP-*N*-acetylglucosamine induced a glutamine-binding site on glucosamine synthetase.

Desensitization. Many regulatory enzymes have been observed to lose the modifier capacity while retaining the catalytic function. This property, desensitization, can be induced by several different methods, both physical and chemical. In this paper desensitization has been induced by two techniques representative of these categories.

The physical method employed was differential thermal inactivation of preparation II in the presence of 1 mM-fructose 6-phosphate at pH 7.5. The temperature-dependence of the first-order rate constants for the inactivation process of the active site is shown in Fig. 5. From this was calculated an enthalpy change of 75 kcal/mol, which is in the range

expected for a process of protein denaturation. An optimum working temperature of 46°C was chosen for subsequent experiments, and samples were removed at intervals over a 60 min period and assayed for total activity, control ratio and modifier ratios (see the Materials and Methods section).

The control ratio for UDP-*N*-acetylglucosamine increased approximately linearly over the 60 min period, signifying a decreased interaction between the UDP-*N*-acetylglucosamine and catalytic sites (Fig. 6a). The effect of the thermal denaturation on the interaction between the AMP-, glucose 6-phosphate- or UTP-binding sites and the UDP-*N*-acetylglucosamine-binding site is shown in Fig. 6(b). This figure expresses these interactions in terms of the enzymic response to the modifier as measured by the modifier ratio. The variation in ratios for AMP and glucose 6-phosphate disclosed that initially glucosamine synthetase was desensitized to these two modifiers faster than to UDP-*N*-acetylglucosamine. After 30 min these modifier ratios became constant, suggesting equal rates of destruction of the UDP-*N*-acetylglucosamine- and modifier-binding sites. The corresponding ratio for the influence of UTP on the feedback-inhibitor-binding site displayed insignificant variation over the incubation period.

The chemical desensitization procedure employed inactivation of thiol groups with 4-chloromercuribenzoate. Preincubation of the enzyme (preparation II) in the presence of 1 mM-fructose 6-phosphate at pH 7.5 and 37°C with this thiol-

Fig. 6. Effect of thermal denaturation on the sensitivity of glucosamine synthetase to its modifiers. The enzyme (1.2 mg of protein/ml) was maintained at 46°C in buffer described for Fig. 5. Samples were removed at intervals for assay for total activity and control and modifier ratios as described in the text. (a) $\circ$, Total activity; Δ , activity in the presence of 0.1 mM-UDP-N-acetylglucosamine; $\square$, control ratio. (b) Modifier ratios for: $\bullet$, AMP (2.0 mM); $\blacktriangle$, glucose 6-phosphate (1.5 mM); $\blacksquare$, UTP (2.5 mM).

Fig. 7. Effect of 4-chloromercuribenzoate on the response of glucosamine synthetase to its modifiers. The enzyme was incubated with the stated concentration of 4-chloromercuribenzoate in the standard assay. The total activity and control and modifier ratios were determined as described in the text. (a) $\circ$, Total activity; Δ , activity in the presence of 0.1 mM-UDP-N-acetylglucosamine; $\square$, control ratio. (b) Modifier ratios for: $\bullet$, AMP (2.0 mM); $\blacktriangle$, glucose 6-phosphate (1.5 mM); $\blacksquare$, UTP (2.5 mM).

blocking reagent showed that reaction with the thiol groups was virtually instantaneous and irreversible. The enzyme was assayed for total activity and control and modifier ratios at a series of 4-chloromercuribenzoate concentrations (0–100 μM). The results are shown in Figs. 7(a) and 7(b). The

control ratio increased over the range of mercurial concentration 0–25 μM but at concentrations greater than 25 μM the change in the ratio was not so marked. Low 4-chloromercuribenzoate concentrations decreased the modifier ratios for AMP and glucose 6-phosphate, signifying an enhancement in

the interaction between these modifiers and the UDP-*N*-acetylglucosamine-binding site. However, as the mercurial concentration was increased the ratios tended to unity, showing a complete suppression of the action of AMP and glucose 6-phosphate. With UTP there was a decrease in the modifier ratio at low concentrations of 4-chloromercuribenzoate, but above 50 μM the ratio returned to the value obtained in the absence of the mercurial.

Glutamine-binding site. The glutamine-binding site was investigated by using a series of potential analogues. The effects of these analogues were investigated at pH 7.5 on the enzyme as prepared by preparation II in the standard incubation.

Asparagine was inhibitory with K_i 1.5×10^{-2} M as determined by the plot of $1/v$ versus $[I]$ (Webb, 1963).

O-Diazooacetyl-L-serine (azaserine) has been found to behave as a glutamine analogue towards many amide aminotransferases (Meister, 1962) including glucosamine synthetase (Danishefsky & Deutsch, 1968). In the present studies, with saturating fructose 6-phosphate and 4 mM-glutamine, the maximum inhibition was 65%, which was achieved with 5 mM-azaserine; any increase in inhibitor concentration did not decrease the activity further. It has been reported that azaserine inactivates enzymes by alkylation (Hartman, 1963), but in the present studies no irreversible inhibition was detected during a 3 h incubation either with or without the substrates present. The inhibition by azaserine was non-competitive for fructose 6-phosphate and the dependence of $1/V$ on the inhibitor concentration was not linear. Two effects were noted with glutamine as the variable substrate: at low substrate concentrations azaserine behaved uncompetitively, whereas at concentrations approaching saturation the analogue displayed a competitive pattern (Fig. 8). Both phases of the action exhibited non-linear dependence on the concentration of azaserine.

L- α -Amino- β -ureidopropionic acid (albizzin), in which a $-\text{CH}_2-$ group of glutamine is replaced by $-\text{NH}-$, displayed the same inhibitory characteristics as azaserine although it was not as potent.

Although 6-diazo-5-oxo-L-norleucine had previously been found to be a potent inhibitor (Ghosh *et al.* 1960; Bates & Handschumacher, 1969), the *N*-acetyl- α -amino derivative (Duazomycin A) at 5 mM had no effect on the enzyme when tested in the present studies. This was in contrast with its noted action *in vivo* (Bates & Handschumacher, 1969).

Other possible glutamine analogues that did not affect the activity at 10 mM final concentration were *S*-carbamoyl-L-cysteine, L-methionine-DL-sulphoxide, γ -methyl glutamate, γ -ethyl glutamate,

Fig. 8. Inhibition of glucosamine synthetase by azaserine with glutamine as the variable substrate. The velocity was determined in the standard incubation with saturating fructose 6-phosphate and the stated concentration of azaserine. $\circ$, Control; Δ , 1.5 mM-azaserine; $\square$, 4.0 mM-azaserine.

α -amino-*n*-butyric acid, norleucine, lysine and DL-5-hydroxylysine.

DISCUSSION

It is the purpose of this discussion to correlate the evidence presented in this paper with that reported in the preceding papers (Winterburn & Phelps, 1971a,b). Unless otherwise stated, all of the kinetic effects quoted in this discussion were derived from these two preceding papers. While this work was in progress a report was published on glucosamine synthetase (Bates & Handschumacher, 1969). Unfortunately the paper, which approached the mechanistic problem from the novel situation of using an alkylating substrate analogue, 6-diazo-5-oxo-L-norleucine, is not fully complementary to the kinetic and stability studies reported here. Since the authors used glucose 6-phosphate as a stabilizing agent their conclusions apply to the glucose 6-phosphate-modified enzyme. In the present studies each ligand was assessed independently before commitment to more complex mixtures.

The effect of the substrates on stability revealed that glutamine is weakly bound by the free enzyme (apparent binding constant 7.5×10^{-2} M) whereas fructose 6-phosphate is strongly bound at two or more sites with differing binding constants. This is in agreement with a sequential addition of substrates, fructose 6-phosphate binding before glutamine, a result supported by the kinetic analysis.

A number of metabolites exert influence on the activity of glucosamine synthetase. Two features of these effects are significant: that there is a large number of these modifiers and that the action of one modifier is often dependent on the presence of another. In the absence of substrates UDP-*N*-acetylglucosamine stabilizes the enzyme with an apparent binding constant of $3.5 \times 10^{-5} \text{ M}$, which compares with the kinetically derived K_i of $7 \times 10^{-5} \text{ M}$. UDP-*N*-acetylglucosamine is essentially an uncompetitive inhibitor for glutamine but competitive for fructose 6-phosphate. Since the substrate binding order is fructose 6-phosphate followed by glutamine, this implies that UDP-*N*-acetylglucosamine binds preferentially to the free enzyme. However, the existence of a small but finite value for the K_i of the $V_{\text{Fru-6-P}}$ term indicates that UDP-*N*-acetylglucosamine combines to a smaller extent with other enzyme forms. Although the free enzyme was not significantly stabilized by glutamine, in the presence of UDP-*N*-acetylglucosamine this substrate did increase the stability of the enzyme. Therefore a glutamine-binding site is induced by UDP-*N*-acetylglucosamine that is not apparent in the absence of the feedback inhibitor.

Kinetically AMP only demonstrates its effects in the presence of UDP-*N*-acetylglucosamine. In agreement the enzyme apparently only binds AMP when the feedback inhibitor is also present. The apparent binding constants for the AMP+UDP-*N*-acetylglucosamine combination are approx. 10^{-5} M whether calculated kinetically or from stability data. This implies that the feedback inhibitor induces an AMP-binding site and, since AMP lowers the K_i for UDP-*N*-acetylglucosamine, the occupation of this site by AMP in turn increases the binding of the UDP-*N*-acetylglucosamine.

Glucose 6-phosphate stabilizes the enzyme against inactivation. From this it is deduced that glucose 6-phosphate binds to the free enzyme with an apparent binding constant of $4.4 \times 10^{-4} \text{ M}$. This protective action is in accord with the use of glucose 6-phosphate by some workers to stabilize glucosamine synthetase preparations (Kornfeld, 1967; Bates & Handschumacher, 1969). Kinetically it is observed that glucose 6-phosphate exerts only a slight effect on the catalytic activity, its prime action being a modification of the binding of UDP-*N*-acetylglucosamine. This is borne out by the binding studies, where the apparent binding constant for UDP-*N*-acetylglucosamine is lowered in the presence of glucose 6-phosphate. Although glucose 6-phosphate presumably induces a conformation change in the environment of the UDP-*N*-acetylglucosamine-binding site that results in a stronger binding of the feedback inhibitor, and this in turn affects the catalytic function of the enzyme, there is no reciprocal effect by UDP-*N*-acetyl-

glucosamine on the glucose 6-phosphate binding site. This is in contrast with the synergistic effect noted with AMP.

UTP has no kinetic effect on the enzyme, nor does its presence affect the stability of the enzyme. It is concluded that UTP does not bind to the free enzyme. However, in the presence of the UDP-*N*-acetylglucosamine-enzyme complex the effect of UTP, as measured in these stability studies, was to increase the K_i for the feedback inhibitor corresponding to a dissociation of the inhibitor from the enzyme. This is in agreement with the kinetically observed behaviour of UTP.

On the basis of inactivation experiments with 6-diazo-5-oxo-L-norleucine it had been concluded that UDP-*N*-acetylglucosamine does not bind at the glutamine-binding site (Bates & Handschumacher, 1969). The present work confirms and extends this finding by demonstrating that the feedback inhibitor is bound at a site distinct from the catalytic one. The evidence in support of this contention is: (a) 4-chloromercuribenzoate causes a decrease in the potency of the inhibition, possibly by reacting with a thiol group either at a binding site or at one involved in the transmission of a conformation change between sites, (b) the induction of conformational change by thermal denaturation affects the inhibition by UDP-*N*-acetylglucosamine more than the catalytic activity, and (c) prolonged contact between the enzyme and DEAE-cellulose during purification can result in the preparation of an enzyme that, though catalytically active, is devoid of inhibition by UDP-*N*-acetylglucosamine (also noted by Kornfeld, 1967).

During thermal or chemical denaturation the sensitivity towards glucose 6-phosphate or AMP declines faster than that towards UDP-*N*-acetylglucosamine, and $100 \mu\text{M}$ 4-chloromercuribenzoate completely desensitizes the enzyme to these two effectors. Similarly two anti-rheumatic agents, phenylbutazone and 4-isobutylphenylpropionic acid (ibuprofen), which have no effect on the action of UDP-*N*-acetylglucosamine, decrease the potency of glucose 6-phosphate and AMP (P. J. Winterburn, unpublished work). The evidence is compatible with the glucose 6-phosphate- and AMP-binding sites being distinct from both the catalytic and the UDP-*N*-acetylglucosamine-binding sites. The kinetic evidence shows that the modifications by glucose 6-phosphate and AMP of the inhibition by UDP-*N*-acetylglucosamine are essentially identical. Similarly the desensitization experiments show that throughout the range of denaturing conditions employed there is a close correlation between the actions of glucose 6-phosphate and AMP. It is suggested that the conformational or environmental changes induced by these two effectors are very similar. However, the extension of this hypothesis

that these two sites have a common location seems improbable, since the free enzyme will bind glucose 6-phosphate but not AMP and also, unlike that of AMP, the binding of glucose 6-phosphate is unaffected by UDP-*N*-acetylglucosamine.

Although the evidence is not conclusive, the results may be interpreted in terms of a common site for the binding of UTP and UDP-*N*-acetylglucosamine. The evidence may be summarized: (a) thermal denaturation results in parallel losses in sensitivity to UTP and UDP-*N*-acetylglucosamine, (b) after an initial alteration in effect 4-chloromercuribenzoate densensitizes the enzyme to UTP and UDP-*N*-acetylglucosamine at equal rates, (c) neither of the two anti-rheumatic agents affect the response of the enzyme to UDP-*N*-acetylglucosamine or to UTP (P. J. Winterburn, unpublished work) and (d) the kinetic and stability behaviour of UTP is consistent with a competitive inhibition for UDP-*N*-acetylglucosamine. If UTP and UDP-*N*-acetylglucosamine compete for a common site, then UTP activates by being an inhibitor of an inhibitor.

Kornfeld (1967) also concluded that the catalytic and UDP-*N*-acetylglucosamine-binding sites were distinct. However, since her preparations contained phosphoglucose isomerase activity many of the results were compatible with an inactivation of the glucose 6-phosphate-binding site rather than the UDP-*N*-acetylglucosamine-binding one. The present work demonstrates that the response to glucose 6-phosphate is more sensitive to denaturation than that for the feedback inhibitor. Also the effect of pH on the control ratio was consistent with a depletion of the reactive inhibitor species rather

than a differential action of the hydrogen ion concentration at two distinct loci.

The binding order of the modifiers is depicted diagrammatically in Scheme 1, where the permitted transitions are shown as solid arrows, prohibited ones as straight lines and those still under investigation are broken arrows. This scheme has been simplified since it takes no account of the presence of substrates, although the kinetic evidence suggests that the modifiers compete for the enzyme form that binds fructose 6-phosphate rather than that binding glutamine (as substrate).

The analogue results imply that the binding of glutamine at the catalytic site involves the α -amino and an un-ionized terminal nitrogen probably close to a carbonyl function. The composition of the chain linking the two functional regions of the molecule is relatively unimportant. The requirement for the amide portion is shown by: (a) amino acids with aliphatic side chains (norleucine and α -amino-*n*-butyric acid) or ionized ω groupings (glutamate, lysine and 5-hydroxylysine) do not inhibit, (b) analogues having a carbonyl function close to an uncharged nitrogen, i.e. asparagine, albizzin, azaserine and 6-diazo-5-oxo-L-norleucine (Bates & Handschumacher, 1969), are inhibitory whereas those without a nitrogenous group (γ -methyl glutamate, γ -ethyl glutamate and methionine sulphoxide) do not inhibit, and (c) un-ionized ammonia is non-inhibitory. The binding of the α -amino-carboxylate portion requires concomitant binding of the amide-type function as shown by the absence of inhibition by α -amino-*n*-butyric acid and norleucine. However, that this part of the

Scheme 1.

Scheme 2.

E, Enzymes; F, enzyme + fructose 6-phosphate at modifier site.

Scheme 3.

substrate is involved is demonstrated by the failure of Duazomycin A to inhibit although its deacetylated form, 6-diazo-5-oxo-L-norleucine, is a potent inhibitor. Further, since Bates & Handschumacher (1969) deduced that Duazomycin A inactivated glucosamine synthetase *in vivo*, presumably this compound is deacetylated to 6-diazo-5-oxo-L-norleucine before becoming active. The non-specific binding of the $-\text{CH}_2-\text{CH}_2-$ group is proposed because azaserine and albizzin, which possess $-\text{CH}_2-\text{O}-$ and $-\text{CH}_2-\text{NH}-$ respectively, can be bound and are inhibitory. In possible contradiction is the lack of inhibitory action of *S*-carbamoyl-cysteine although this compound is structurally similar to glutamine.

All the evidence supports the primary operation of an ordered pathway of substrate addition leading to a ternary complex, with fructose 6-phosphate binding before glutamine. An alternative pathway with a reversed order of substrate addition may operate under certain circumstances, the evidence being: (a) glutamine does bind weakly, (b) in the presence of UDP-*N*-acetylglucosamine substrate inhibition is observed and (c) the uncompetitive inhibition by azaserine and albizzin at low glutamine concentrations is interpreted as a binding to the free enzyme, presumably at the glutamine-binding site. Thus a complete scheme of substrate additions is shown in Scheme 2. This is similar to a model proposed on theoretical grounds by Ferdinand (1966). The scheme relies on the operation of a preferred pathway, via the enzyme-fructose 6-phosphate complex, which has a higher overall velocity than the alternative pathway and also that the breakdown of ternary complex into products is not rate-limiting. This latter point distinguishes this model from a rapid-equilibrium random Bi Bi mechanism. The kinetic patterns predicted by Ferdinand (1966) for this model are in exact agreement with those observed with glucosamine synthetase in the presence of UDP-*N*-acetylglucosamine. An alternative possibility that the feedback inhibitor leads to an abortive dead-end complex, enzyme-glutamine-UDP-*N*-acetylglucos-

amine, is unlikely since a dead-end inhibitor should generate linear dependency of inhibition with inhibitor concentration. This is not observed. The residual activity of 5–10% maximal velocity that is present even when UDP-*N*-acetylglucosamine is saturating may represent the activity of the fully induced alternative pathway. A prediction derived from this model is that if the enzyme is in the enzyme-fructose 6-phosphate form substrate inhibition by glutamine should not occur. This may be tested kinetically by extrapolating to saturating fructose 6-phosphate. The experimental findings are in accord with the prediction. UDP-*N*-acetylglucosamine partially protects the enzyme from alkylation by 6-diazo-5-oxo-L-norleucine (Bates & Handschumacher, 1969), suggesting that UDP-*N*-acetylglucosamine does alter the conformation of the glutamine-binding site. Therefore it is concluded that UDP-*N*-acetylglucosamine inhibits, in part, by modifying the catalytic site so that the glutamine-binding site becomes unmasked and thus introduces an alternative reaction pathway.

These conclusions may be compared with suggestions for other glutamine aminotransferases. Thus an ordered addition with the amino acid binding second has been proposed for the chicken liver phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14) (Hartman, 1963), although the same enzyme from pigeon liver displays random addition (Caskey, Ashton & Wyngaarden, 1964) in common with the phosphoribosyl-formylglycine amidine synthetase (EC 6.3.5.3) (Mizobuchi & Buchanan, 1968).

Some of the effects of fructose 6-phosphate are not compatible with the simple model proposed. These phenomena, e.g. the complex effect of fructose 6-phosphate on the stability, are in accord with at least two fructose 6-phosphate-binding sites. These two could represent a catalytic site and a modifier or stabilizer site. This latter site would have a long time-constant for fructose 6-phosphate binding both at 4°C and 37°C, since although a 24h incubation at 4°C does convert preparation I into an

enzyme form similar to preparation II an incubation at 37°C for 1 h does not. Scheme 3 offers an explanation of the data. All transitions from E to F are slow. F has a higher V than E and has a lower K_m for fructose 6-phosphate although that for glutamine is the same. This model accounts not only for the difference in the enzyme as prepared by the two methods but also for the biphasic stabilization effect. The initial increase in activity is by formation of F with its higher V , but subsequently F, like E, denatures, so generating the second phase.

Davidson (1966) suggested that the complex reaction may be catalysed by two enzymes acting sequentially. However, the high degree of purity of the glucosamine synthetase almost certainly precludes this possibility. It may be significant that the glutamine aminotransferases that can utilize ammonia and exhibit alkylation by azaserine are those that require the participation of either a phosphorylated enzyme intermediate or an enzyme-AMP-‘amine acceptor’ complex. It was proposed for one enzyme in this category, phosphoribosyl-formylglycine amidine synthetase (Mizobuchi, Kenyon & Buchanan, 1968), that the concerted action of a nucleophilic attack by a thiol group labilizing the amine and the electron-withdrawing effect of the phosphorylated enzyme intermediate induced the amination of the carbonyl function. This type of mechanism is not applicable to glucosamine synthetase because there is no pyrophosphate cleavage and the 4-chloromercuribenzoate inhibition is not consistent with the localization of a labile thiol group at the active site.

Thus the enzyme-catalysed formation of glucosamine 6-phosphate by glucosamine synthetase possesses few properties in common with other glutamine aminotransferases. However, there are several similarities between this reaction and the Lobry de Bruyn-Alberda van Eckenstein transformations as catalysed by phosphoglucose isomerase (EC 5.3.1.9) and phosphomannose isomerase (EC 5.3.1.8) (Dyson & Noltman, 1968; Gracy & Noltman, 1968). These similarities are: (a) ketose to aldose isomerization with change in ring size, (b) the observable ionizing groups have apparent pK values of 6.95 and 8.2; although it is dangerous to ascribe functional groups on the basis of pK without knowledge of heat of ionization or chemical modification data, the lower could correspond to a histidine and the one at 8.2, being too acidic for lysine, tyrosine or thiol could be an α -amino group, as has been suggested for phosphomannose isomerase, (c) the fructose 6-phosphate

binding involves the higher pK grouping and this is in its acidic form. The foregoing facts imply that glucosamine synthetase has a greater affinity with the isomerases than with the glutamine aminotransferases.

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