Kinetic Characterization of *N*-Acetyl-D-glucosamine Kinase from Rat Liver and Kidney

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1. Under normal assay conditions the *N*-acetyl-D-glucosamine kinases from rat liver and kidney show a pH-dependent lag phase before reaching a steady state, which is probably due to reversible dissociation of the dimeric enzyme. 2. The enzyme catalyses the phosphorylation of *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine and D-glucose at pH 7.5, with apparent K_m values of 0.06, 0.95 and 600 mM respectively for the enzyme from liver and 0.04, 1.0 and 410 mM respectively for the kidney enzyme. It is strongly inhibited by ADP. 3. The interaction between the enzymes and acceptor substrates shows non-Michaelian kinetics with respect to *N*-acetyl-D-glucosamine but normal behaviour towards *N*-acetyl-D-mannosamine and D-glucose. 4. Both *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine inhibit the phosphorylation of D-glucose; this inhibition appears to be mixed in character. 5. The facts that the enzymes catalyse the phosphorylation of *N*-acetyl-D-mannosamine and D-glucose do not detract from the designation of the enzymes as *N*-acetyl-D-glucosamine kinase. Phosphorylation of glucose *in vivo* by these kinases is unlikely.

Low activities of *N*-acetyl-D-glucosamine kinase (EC 2.7.1.59) are present in a number of mammalian tissues. In the preceding paper (Allen & Walker, 1980) we describe the isolation of the enzyme from the soluble fraction of rat liver and kidney in good yield and very much higher specific activity (approx. 40 units/mg of protein) than previously reported for the mammalian enzyme. In particular, our preparations contained no other related kinase activity. The absolute amount of the enzyme present in these tissues is nevertheless very low. We now report the basic kinetic characterization of the enzyme in liver and kidney.

Materials and Methods

Enzyme preparations

N-Acetyl-D-glucosamine kinase was prepared from rat kidney and liver tissue as described in the preceding paper (Allen & Walker, 1980). Each preparation contained only one band of kinase activity (see Allen & Walker, 1980) and the specific activities were respectively 42 and 39 units/mg of protein, where 1 unit is the activity that catalyses the phosphorylation of $1\mu\text{mol}$ of *N*-acetyl-D-glucosamine/min at 32°C. The enzyme preparations were diluted with the assay buffer (see below) on a small column of Sephadex G-25.

Enzyme-assay procedures

In most experiments the activity of *N*-acetyl-D-glucosamine kinase was assayed by measuring ADP formation as described in detail (Allen & Walker, 1980). This procedure was also convenient for following the activity of the enzymes upon other substrates (glucose or *N*-acetyl-D-mannosamine). Care was taken to ensure that the other enzymes in this coupled assay were not limiting under any of the conditions used. The activity recorded was proportional to the amount of *N*-acetyl-D-glucosamine kinase added throughout the range of activities employed.

In the experiments on the inhibition by *N*-acetyl-D-glucosamine or *N*-acetyl-D-mannosamine of glucose phosphorylation catalysed by the *N*-acetyl-D-glucosamine kinase, the rate of glucose 6-phosphate formed was measured by the standard glucokinase/hexokinase assay procedure. The incubation medium contained, in a final volume of 1 ml: 150 mM-KCl, 20 mM-triethanolamine buffer, pH 7.5, 10 mM-MgCl₂, 5 mM-ATP, 0.2 mM-NADP⁺ and 0.4 unit of glucose 6-phosphate dehydrogenase. That the glucose 6-phosphate dehydrogenase added has some glucose dehydrogenase activity is well documented (Kuby & Noltman, 1966); this has a high K_m with respect to glucose. The rates of glucose 6-phosphate

formation at each glucose concentration were therefore measured in the presence of the added glucose 6-phosphate dehydrogenase but before the addition of the *N*-acetyl-D-glucosamine kinase; the latter was then added and the rate of glucose phosphorylation due to the latter determined by difference. Because the glucose dehydrogenase activity was substantial at the higher glucose concentrations, the accuracy of the results obtained by this procedure was lower than with the ADP assay.

Kinetic constants could be obtained by eye from plots of s/v against s , where s is the concentration of the varied substrate and v is the measured rate of the linear portion of progress plots, but were calculated by linear-regression analyses of the data.

Materials

These have been given previously (Holroyde *et al.*, 1976; Allen & Walker, 1980).

Results and Discussion

Fig. 1 demonstrates with the ADP assay a time lag in achieving a linear rate for the reaction cata-

Fig. 1. Effect of pH on velocity-time progress curves of *N*-acetyl-D-glucosamine kinase activity

The reaction mixture contained in a total volume of 1 ml: 5 mM-ATP, 10 mM-MgCl₂, 150 mM-KCl, 2 mM-*N*-acetyl-D-glucosamine, 0.4 mM-NADH, 2.5 mM-phosphoenolpyruvate, 1.25 units of pyruvate kinase, 3.25 units of lactate dehydrogenase, a fixed amount (approx. 0.01 unit) of rat liver enzyme and buffer solution consisting of 0.1 M-triethanolamine hydrochloride plus 0.1 M-diethanolamine hydrochloride adjusted with KOH to pH 7.5 (○), pH 6.5 (●) or pH 9.0 (□).

lysed by the liver *N*-acetyl-D-glucosamine kinase. Control experiments revealed that this lag was not due to any limitation of the coupled-assay system; addition of a small amount of ADP to the reaction mixture resulted in an immediate rapid oxidation of NADH. The lag was greater at pH 6.5 and 9.0 (Fig. 1) than at the normal assay pH of 7.5. This made it difficult to obtain an accurate pH-activity profile for the enzyme. The normal procedure adopted was to take the rate between 5 and 10 min after the start of the reaction. Fig. 2 shows a pH profile for the liver enzyme; that for the *N*-acetyl-D-glucosamine kinase from kidney was essentially identical. Both enzymes showed maximum activity around pH 7.4, were relatively stable at pH values as low as 6.2, but rapidly lost activity above pH 8.5.

Some evidence was given in the preceding paper (Allen & Walker, 1980) that *N*-acetyl-D-glucosamine kinase may be a dimer. A possible explanation of the curved reaction time course is that the dimer dissociates when the enzyme is greatly diluted on addition to the reaction mixture, that this dissociation is en-

Fig. 2. Effect of pH on activity of *N*-acetyl-D-glucosamine kinase from rat liver

The conditions were the same as in Fig. 1. The pH of the incubation mixture was adjusted to the required pH with KOH. The velocities were calculated from the linear portions of the progress curves as illustrated in Fig. 1.

Fig. 3. Effect of enzyme concentration on the time taken for the reaction to reach steady-state conditions

The standard assay conditions as in Fig. 1 at pH 9.0 and the rat liver enzyme (0.01 unit) were used. The time taken was estimated as described in the text.

hanced by high pH and that reassociation occurs on addition of substrate. Fig. 3 shows the effect of enzyme concentration upon the time taken to reach a linear reaction rate. The latter was estimated by extrapolating the linear portion of a reaction progress plot to the time axis and measuring the time (in min) between the actual start of the reaction and this intercept. The delay in reaching linearity decreases as the enzyme concentration is increased (Fig. 3). A similar result was obtained for the kidney enzyme. This behaviour can be interpreted in terms of a dissociating system. Taken together with the behaviour during gel filtration and the molecular-weight evidence for the dimeric nature of the enzyme (Allen & Walker, 1980), it seems reasonable to deduce that *N*-acetyl-D-glucosamine kinase can undergo reversible dissociation, which is influenced by dilution and pH, to form monomers having a lower specific activity than that of the dimer. Further examination of this interesting phenomenon would be dependent upon the availability of substantially larger amounts of pure enzyme.

Effects of Mg^{2+} ions and ATP

As for other kinases (Walker, 1966), the liver and kidney *N*-acetyl-D-glucosamine kinases require Mg^{2+} ions as cofactors, and no inhibition by excess Mg^{2+} was observed. The results in Figs. 4(a) and 4(b) show the extrapolated lines passing through the origin of the $[MgCl_2]/v$ versus $[MgCl_2]$ plots. The departure from linearity of the observations occurred at the concentration (5 mM) of ATP present, indicating an optimum $[Mg^{2+}]/[ATP]$ ratio of 1. This was true with both *N*-acetyl-D-glucosamine and glucose as substrate. In studies on less-pure enzymes the required ratio has been given as 2 for the *N*-acetyl-D-glucosamine kinase from pig spleen (Datta, 1970) and 0.75 for the enzyme from human gastric mucosa (Gindzienski *et al.*, 1974). We observed no evidence of the necessity for excess Mg^{2+} over ATP with our preparations such as is seen for many enzymes (e.g. Morrison & Ebner, 1971; Elliot & Tipton, 1974).

Plots of $[ATP]/v$ against $[ATP]$ at a fixed Mg^{2+} ion concentration of 10 mM gave straight lines (Fig. 5), and indicated a K_m for $MgATP^{2-}$ of 0.23 and 0.10 mM with *N*-acetylglucosamine as acceptor substrate for the liver and kidney enzymes respectively, and 0.65 and 0.68 mM respectively with glucose as the other substrate. These values are somewhat lower than the 1.8 mM reported for the pig spleen enzyme (Datta, 1970) and 3.0 mM for the human gastric-mucosa enzyme (Gindzienski *et al.*, 1974), but similar to the value of 0.48 mM given for the partially purified rat liver enzyme (Saeki *et al.*, 1971).

Fig. 4. Effect of concentration of $MgCl_2$ upon velocity. The standard assay conditions were used for (a) rat liver enzyme and (b) rat kidney enzyme, except as indicated below. A fixed amount of the enzyme preparation (0.005 and 0.002 unit respectively) was added. The substrates employed (final concns.) were: O, 600 mM-glucose; ●, 2 mM-*N*-acetyl-D-glucosamine.

Dependence of the rate on the acceptor-substrate concentration

Plots of $[N\text{-acetyl-D-glucosamine}]/v$ against $[N\text{-acetyl-D-glucosamine}]$ at a fixed $MgATP^{2-}$ concentration (5 mM) for both the liver and kidney enzymes gave straight lines (not illustrated), except that a very slight upward curvature was noted at very low concentrations of *N*-acetyl-D-glucosamine. K_m values of 0.06 mM- and 0.04 mM-*N*-acetyl-D-glucosamine were calculated for the liver and kidney enzymes respectively. These values compare well with that of 0.06 mM reported for the partially purified liver enzyme (Saeki *et al.*, 1971), but are lower than those for the enzyme from other tissues (Gindzienski *et al.*, 1974; Datta, 1970). Because of the slight curvature, the data were expressed as a Hill plot of $\log[v/(V_{max} - v)]$ against $\log[N\text{-acetyl-D-glucosamine}]$, where V_{max} is the apparent maximum velocity given by extrapolation of the s/v versus s plots (Fig. 6). A Hill coefficient (h) as given by the slope of these lines, was found to be 1.54 for

the kidney enzyme and 1.27 for the liver enzyme. The likely dimeric nature of the enzymes and the suggested effect of substrate upon its reversible

Fig. 5. Effect of concentration of ATP upon velocity. The standard assay conditions were used for (a) rat liver enzyme and (b) rat kidney enzyme as in Fig. 4. The concentration of MgCl_2 was 10 mM and the ATP concentration was varied as shown. The substrate concentrations were: O, 600 mM-glucose; ●, 2 mM-N-acetyl-D-glucosamine.

Fig. 6. Hill plot of dependence of velocity upon N-acetyl-D-glucosamine concentration.

The standard assay conditions were used, except that the concentration of N-acetyl-D-glucosamine was varied. The plots are given for rat liver enzyme (O) and rat kidney enzyme (●) as in Fig. 4. The Hill coefficients (h) are 1.27 and 1.54 respectively.

dissociation (see above) can explain this small degree of co-operativity. The N-acetyl-D-glucosamine kinase from pig spleen also displays co-operativity with respect to substrate with a Hill coefficient of 1.66 (Datta, 1971).

The phenomenon was not observed, however, with glucose as substrate. Although a comparatively poor substrate for the enzyme (Allen & Walker, 1980), we were interested to examine the phosphorylation of glucose by it because of the possibility that reports [see preceding paper (Allen & Walker, 1980)] of 'glucokinase' activity in non-hepatic tissues was due to N-acetyl-D-glucosamine kinase. When the coupled assay that measures glucose 6-phosphate formation was used, plots of $[\text{glucose}]/v$ against $[\text{glucose}]$ (Fig. 7) were straight lines, indicating a K_m for glucose of 600 and 410 mM respectively for the liver and kidney enzymes. The absence of any sign of substrate co-operativity for glucose was confirmed by Hill plots that gave a value for h of 1.0. The same results were obtained by using the ADP-formation assay (as had, of course, to be used with N-acetyl-D-glucosamine as substrate). The V_{max} with glucose as substrate was 53% of that for N-acetyl-D-glucosamine for the kidney enzyme; the corresponding result for the liver enzyme was 73%.

Although electrophoretic studies on our enzyme preparations indicated the presence of only one kin-

Fig. 7. Effect of glucose concentration upon the rate of glucose 6-phosphate formation catalysed by N-acetyl-D-glucosamine kinase.

The formation of glucose 6-phosphate was measured by coupling this to the reduction of NADP^+ as described in the Experimental section. O, Rat liver enzyme; □, rat kidney enzyme. Conditions were as in Fig. 4.

ase activity in each (Allen & Walker, 1980), the fact that a major contaminant that had come through an affinity-chromatography procedure along with the enzyme was present, left open a doubt as to whether the phosphorylation of *N*-acetyl-D-glucosamine and glucose was due to the same enzyme. The effect of *N*-acetyl-D-glucosamine upon the glucose-phosphorylating activity was therefore examined using the assay of glucose 6-phosphate formation. The plots of $1/v$ against $[N\text{-acetyl-D-glucosamine}]$ at three different glucose concentrations for both liver and kidney enzymes (Fig. 8) suggested that *N*-acetyl-D-glucosamine was a non-competitive inhibitor with respect to glucose, with a K_i of 0.04 mM for the liver enzyme and 0.02 mM for the kidney enzyme; these values are very similar to the K_m values for *N*-acetyl-D-glucosamine given above. Such Dixon plots of $1/v$ against inhibitor concentration do not distinguish too well between certain forms of inhibition and, as shown by Cornish-Bowden (1974), plots of s/v against inhibitor con-

centration can give complementary information. Replotting of the data in Fig. 8 in that manner gave straight lines intersecting below the abscissa, which is indicative of mixed inhibition (Cornish-Bowden, 1974).

N-Acetyl-D-mannosamine is also a good substrate for our liver and kidney enzyme preparations and this activity is almost certainly due in fact to the same enzyme. Plots (not shown) of $[N\text{-acetyl-D-mannosamine}]/v$ against $[N\text{-acetyl-D-mannosamine}]$ for the liver and kidney enzymes at 5 mM- MgATP^{2-} were straight lines, indicating K_m values of 0.95 and 1.0 mM respectively and $V_{\max}$ values 100 and 83% respectively of those with *N*-acetyl-D-glucosamine. Unlike for *N*-acetyl-D-glucosamine, there was no evidence of any co-operativity with respect to *N*-acetyl-D-mannosamine. This was confirmed by presenting the data as Hill plots (Fig. 9), the slopes of which were 1.0 for both the liver and kidney enzymes.

As shown above for *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine was also found to be a mixed inhibitor of glucose phosphorylation (Fig. 10 shows the Dixon plot). The observed K_i values were 0.45 and 0.70 mM for the liver and kidney enzymes respectively. These values are similar to the K_m values. These observations support the conclusion that the phosphorylation of *N*-acetyl-D-glucosamine, glucose and *N*-acetyl-D-mannosamine is due to the same enzyme. The kinase from human gastric mucosa utilizes *N*-acetyl-D-mannosamine at 37% of the rate

Fig. 8. Inhibition of glucose phosphorylation by *N*-acetyl-D-glucosamine

The activities of the *N*-acetyl-D-glucosamine kinases from (a) rat liver and (b) rat kidney as in Fig. 4 were assayed by measuring the formation of glucose 6-phosphate as described in Fig. 7. The concentration of *N*-acetyl-D-glucosamine was varied as indicated, and the concentrations of glucose were 100 mM (O), 300 mM (□) and 600 mM (Δ).

Fig. 9. Hill plot of dependence of velocity upon *N*-acetyl-D-mannosamine concentration

The standard assay conditions were used as in Fig. 6, except that the concentration of *N*-acetyl-D-mannosamine was varied as indicated. O, Rat kidney enzyme (0.0045 unit); □, rat liver enzyme (0.003 unit). The line corresponds to a Hill coefficient (h) of 1.

Fig. 10. Inhibition of glucose phosphorylation by *N*-acetyl-D-mannosamine

The activities of the *N*-acetyl-D-glucosamine kinases from rat liver (open symbols) and rat kidney (closed circles), as in Fig. 9, were assayed as in Fig. 8. The concentration of *N*-acetyl-D-mannosamine was varied as indicated, and the concentrations of glucose were 100 mM (○), 300 mM (□) and 600 mM (△ and ●).

with *N*-acetyl-D-glucosamine (Gindzienski *et al.*, 1974), but *N*-acetyl-D-mannosamine was reported not to be a substrate for the enzyme from pig spleen (Datta, 1970). These workers were, however, measuring uptake of *N*-acetyl-D-mannosamine. For this reason they may not have been fully aware of the strong product inhibition that we have noted using the ADP-formation assay, as evidenced by rapidly decreasing velocities with time. We found that the amount of reaction that occurred depended upon the amount of *N*-acetyl-D-mannosamine present. The reaction actually stopped when about 30% of the substrate available had been utilized. This inhibition was not relieved by the addition of a

further sample of enzyme. These observations suggest that the product, presumably *N*-acetyl-D-mannosamine 6-phosphate, is a strong competitive inhibitor of the reaction.

The enzymes from liver and kidney that we have examined clearly prefer *N*-acetyl-D-glucosamine to glucose as acceptor substrate, and it seems reasonable to conclude that the activity towards glucose is a side issue. The very high K_m for glucose makes it most unlikely that it would be of any physiological relevance in the phosphorylation of glucose *in vivo*, except perhaps in extreme circumstances (Alvares & Nordlie, 1977). The greater than 10-fold difference in K_m values with respect to *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine and the fact that co-operativity is seen with the former but not the latter substrate support the conclusion that the enzyme should be designated *N*-acetyl-D-glucosamine kinase rather than an *N*-acetylhexosamine kinase of broader specificity.

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