A Kinetic Study of a Solubilized Chitin Synthetase Preparation from *Coprinus cinereus*

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SUMMARY

The solubilized chitin synthetase preparation from the stipes of *Coprinus cinereus* is activated by its substrate, UDP-*N*-acetylglucosamine, and by carbohydrates, in particular *N*-acetylglucosamine, diacetylchitobiose and glucose. Analysis of the results of variation in enzyme activity with UDP-GlcNAc concentration by several methods suggested that this substrate is an allosteric activator, with Hill coefficients close to 2 and 4 at concentrations above and below 0.1 mM respectively, and an $[S]_{0.5}$ value of 0.9 mM. *N*-acetylglucosamine lessened but did not abolish the sigmoidal shape of the velocity-substrate concentration plot. The chitin synthetase was inhibited by adenine and uridine nucleotides, and uridine diphosphate was a powerful competitive inhibitor. The enzyme preparation also contained a nucleoside diphosphatase activity and the implications of the removal of the inhibitory UDP formed by the chitin synthetase are considered.

INTRODUCTION

The control of chitin synthesis in the walls of fungi must play an important role during morphogenesis. This process is of particular importance during the development of fruit bodies of the toadstool, *Coprinus cinereus* (Gooday, 1972*a, b*, 1973). The isolation and properties of chitin synthetase (uridine diphosphate-*N*-acetylglucosamine:chitin *N*-acetylglucosaminyltransferase, EC. 2.4.1.16) from the stipes of *C. cinereus* have been discussed in the preceding paper (Gooday & de Rousset-Hall, 1975).

Here we describe experiments designed to investigate the possibility that the activity of the chitin synthetase could be controlled in part by the presence or absence of small molecule effectors. Evidence is presented which suggests that the interaction between chitin synthetase and activating ligands involves allosteric binding sites.

METHODS

Cultures, enzyme preparation and assay. All procedures for the preparation and assay of the solubilized chitin synthetase from *C. cinereus* were as described by Gooday & de Rousset-Hall (1975). All assays were for 5 min at 25 °C, when the reaction rate was always linear. The incubations with varying substrate concentrations each contained the same amount of radioactivity (12.5 nCi).

Investigation of uridine diphosphatase activity. The incubation volume of 20 μ l in a 1.2 ml plastic centrifuge tube contained final concentrations of 63 mM-tris-HCl pH 7.5, 12.5 mM-MgCl₂, 1.25 mM-EDTA (disodium salt) and 25 nCi [4-¹⁴C]uridine-5'-diphosphate ammonium salt (45 mCi/mmol: The Radiochemical Centre, Amersham, Buckinghamshire) with or without the addition of uridine diphosphate (Sigma). The solubilized enzyme preparation

(5 μ l, containing 1 to 2 μ g protein) was added, the tube incubated at 25 °C for 5 min and the reaction stopped by placing the tube in boiling water for 2 min. The contents of the tube were spotted on to the origin of a Merck silica gel F254 t.l.c. plate. The tube was washed three times with 20 μ l of 1 % (v/v) Triton X-100 in 50 % (v/v) aqueous ethanol. The plate was run in ethanol-1 M-ammonium acetate pH 3.8 (7:3, v/v), dried and autoradiographed overnight as described by Gooday & de Rousset-Hall (1975). Transparent adhesive tape was stuck to the t.l.c. plate at the positions of the substrate (R_F 0.10) and radioactive product (R_F 0.44), and left overnight. The silica gel attached to the tape was removed from the glass with a blade, placed in a scintillation vial and counted in a PPO/POPOP scintillant (Gooday & de Rousset-Hall, 1975).

The activity of the UDPase was calculated from the percentage of total counts on the plate which were present in the form of UMP, and was expressed as nmol UMP formed/min/mg protein.

Chemicals. *N*-fluoro- and *N*-iodoacetylglucosamine, and diacetylchitobiose were kindly supplied by Dr P. W. Kent and Dr R. C. W. Berkeley, respectively.

RESULTS

Uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and a divalent metal ion are the sole requirements for chitin synthesis by the solubilized preparation from *Coprinus cinereus* (Gooday & de Rousset-Hall, 1975). When the enzyme was incubated in the presence of different concentrations of UDP-GlcNAc a plot of velocity against UDP-GlcNAc concentration had a sigmoidal appearance (Fig. 1*b*). Very little activity was observed below 0.05 mM-UDP-GlcNAc, and maximal activity (V) was obtained with about 5 mM-UDP-GlcNAc. At low concentrations an increase in UDP-GlcNAc concentration gave very little stimulation in activity, but a similar increase at higher concentrations gave a dramatic stimulation in activity. Thus the substrate activates the enzyme. The particulate preparation (Gooday & de Rousset-Hall, 1975) gave similar results.

The results for velocity against substrate concentration for 14 experiments were taken and the values were normalized, taking the activity at 1.0 mM-UDP-GlcNAc as 100 arbitrary units. The mean value of the velocity at each substrate concentration was taken and used for the subsequent calculations. (As the same substrate concentrations were not evaluated for all the experiments, the mean values were calculated from between 4 and 10 experiments.)

Double reciprocal plots of velocity against substrate concentration were non-linear, having an upward curvature, and so our chitin synthetase preparation does not exhibit Michaelis-Menten kinetics (Fig. 2).

A Hill plot ($\log v/(V-v)$ against $\log [S]$, where v is the velocity at a particular substrate concentration S , and V is the maximum velocity; Atkinson, Hathaway & Smith, 1965) of the normalized results was constructed, using the value for V obtained from the plot of v against $[S]$ for the normalized results. Two intersecting linear regression lines could be drawn through the points (Fig. 3). At UDP-GlcNAc concentrations below 0.1 mM the slope (n) was 3.6. Above this concentration it was 1.7. The $[S]_{0.5}$ value (the concentration giving half maximal velocity) was 0.9 mM.

The Hill coefficient (n) was also calculated by using the plot outlined by Fajszki & Endrenyi (1974) as adapted by J. Jeffery (personal communication), which does not depend on an estimation of V . Pairs of values of substrate concentrations (S_a and S_b) were taken so that the ratio S_b/S_a was a constant arbitrary factor α . The values of the velocity (v_a and v_b)

Fig. 1. Plots of enzyme velocity against UDP-*N*-acetylglucosamine concentration in the absence of effectors (●), in the presence of 20 mM-glucose (○), and in the presence of 5 mM-*N*-acetylglucosamine (■). (a) Results at higher UDP-GlcNAc concentrations; (b) enlarged scale for results at lower UDP-GlcNAc concentrations. Results are from one typical experiment.

corresponding to the substrate concentrations were read from the graph of the normalized results of velocity against substrate concentration in the range of $S_a = 4.0$ to 0.6 mM. When $1/v_b$ was plotted against $1/v_a$ a straight line was obtained (Fig. 4a). The value n can be calculated from the slope by the relationship

$$n = -\frac{\log(\text{slope})}{\log \alpha}.$$

This Hill coefficient was 1.7. When this procedure was repeated for low substrate concentrations ($S_a = 0.1$ to 0.06 mM) the value of n was 4.0 (Fig. 4b). Both of these estimates agree well with the values of n of 1.7 and 3.6 obtained from the Hill plot.

Effect of sugars and amino sugars on chitin synthetase activity

A variety of sugars and amino sugars was tested for their effect on chitin synthetase activity at low and high concentrations of UDP-*N*-acetylglucosamine (Table 1).

Glucose and *N*-acetylglucosamine activated the enzyme at both concentrations of

Fig. 2

Fig. 3

Fig. 2. Lineweaver-Burk plot of the velocity against UDP-*N*-acetylglucosamine concentration in the absence of added effectors. Results normalized from several experiments (see text for method). Bars represent standard deviations of normalized results, for between 4 and 10 readings for each concentration.

Fig. 3. Hill plot of the normalized results for the velocity against UDP-*N*-acetylglucosamine concentration in the absence of added effectors. Two intersecting regression lines, $y = 3.6x + 1.98$ ($r = 0.99$) and $y = 1.7x + 0.04$ ($r = 0.99$) are drawn through the points. Results are from the same experiments as Fig. 2, but a wider range of substrate concentrations have been used.

UDP-GlcNAc. Glucosamine and diacetylchitobiose activated only at low UDP-GlcNAc concentrations, and glucosamine-6-phosphate was inhibitory at low UDP-GlcNAc concentrations.

The effect of optimal concentrations of glucose and *N*-acetylglucosamine was investigated in more detail (Fig. 1). Both these effectors greatly increased the activity of the enzyme at low substrate concentrations, and reduced the concentration below which no activity was detected. However, at UDP-GlcNAc concentrations giving maximal or near maximal activity, *N*-acetylglucosamine had no activating effect, while glucose continued to activate the enzyme. A Lineweaver-Burk plot for the results in the presence of *N*-acetylglucosamine still had an upward curvature, while a Hill plot for the results in the presence of *N*-acetylglucosamine was no longer biphasic. The Hill coefficient, calculated from both the Hill and modified Fajsz and Endrenyi plots, was 1.4.

The specificity of the *N*-acetylglucosamine binding site was investigated using analogues of this substance (Table 2). *N*-fluoroacetyl-D-glucosamine and *N*-iodoacetyl-D-glucosamine are chemical modifications of *N*-acetylglucosamine by halogenation of the acyl group, and have been found to occupy molecular sites specific for *N*-acetylglucosamine (Kent & Dwek, 1971; Kent, Ackers & White, 1970). *N*-fluoroacetylglucosamine activated the enzyme but

Fig. 4. The plot as outlined by Fajsz & Endrenyi (1974) and adapted by J. Jeffery (personal communication) to investigate the kinetics of enzyme-substrate binding: (a) at high substrate concentrations (4.0 to 0.6 mM-UDP-GlcNAc) giving a regression line of $y = 1.81x - 0.004$ ($r = 0.99$); (b) at low substrate concentrations (0.1 to 0.06 mM-UDP-GlcNAc) giving a regression line of $y = 4.09x - 0.307$ ($r = 0.99$). The regression line for the values at high substrate concentration from (a) is drawn on (b) as the dashed line. Results are from the same experiments as Fig. 3.

N-iodoacetylglucosamine and *N*-acetylgalactosamine had no apparent activating or inhibiting effect. However, *N*-iodoacetylglucosamine prevented the activation by *N*-acetylglucosamine and so does appear to bind to the enzyme molecule.

Effect of nucleotides on chitin synthetase activity

Adenine and uridine mono-, di-, and triphosphates were added to enzyme incubations. All six nucleotides inhibited chitin synthetase activity (Table 3), but the uridine nucleotides were more inhibitory. Uridine diphosphate was a powerful inhibitor and its effect has been investigated more fully. Fifty per cent inhibition was obtained with about 0.8 mM-UDP in the presence of 1 mM-UDP-GlcNAc. The K_i was 0.6 mM-UDP as determined from a Hill plot. The $[S]_{0.5}$ value obtained from the Hill plot of v against $[S]$ was increased in the presence of UDP, while V (from the Fajsz & Endrenyi plot) was hardly affected. These results indicate that UDP is a competitive inhibitor, but as detailed below, analysis is complicated by UDP being a product of the chitin synthetase reaction, and by the nucleoside diphosphatase activity in our preparations.

Properties of the nucleoside diphosphatase activity of the chitin synthetase preparation

Thin-layer chromatograms of chitin synthetase assays of particulate and solubilized preparations were routinely run on fluorescent-coated silica gel layers. Viewing these in u.v. light to detect the absorbing spots showed that, in addition to unchanged UDP-GlcNAc, UDP and UMP were always present after the incubation. The UMP has been shown to be the result of the hydrolysis of UDP, by a nucleoside diphosphate phosphohydrolase activity (EC. 3.6.1.6). As UDP is both a product of chitin synthetase activity and a powerful

Table 1. *The effect of sugars and amino sugars on the activity of chitin synthetase from Coprinus cinereus*

Sugar or amino sugar	Concn (mM)	Specific activity at:					
		Low substrate concn (0.1 mM-UDP-GlcNAc)			High substrate concn (1.0 mM-UDP-GlcNAc)		
		Control*	With sugar or amino sugar*	Percentage of control activity	Control*	With sugar or amino sugar*	Percentage of control activity
Glucose	50.0	—	—	—	277	391	141
	5.0	11.1	37.7	340	277	318	115
2-Deoxyglucose	50.0	—	—	—	277	383	138
	5.0	—	—	—	277	345	125
L-Sorbose	50.0	—	—	—	460	477	104
	5.0	—	—	—	460	457	99
Trehalose	50.0	18.6	19.9	108	388	403	104
	5.0	18.6	19.1	103	388	381	98
	0.5	18.6	17.7	96	388	393	101
Glucosamine	50.0	19.3	44.7	231	427	468	110
	5.0	19.3	39.3	218	200	224	112
	0.5	19.3	21.0	109	427	410	96
Glucosamine-6-phosphate	50.0	19.3	6.1	32	427	242	57
	5.0	19.3	14.1	73	427	393	92
	0.5	19.3	16.4	85	691	657	95
<i>N</i> -acetylglucosamine	5.0	5.8	31.9	550	253	351	126
	2.0	10.7	44.3	414	260	328	126
Diacetylchitobiose	2.0	7.0	20.2	289	328	340	104

* Specific activities are expressed as nmol GlcNAc incorporated/min/mg protein. Most values are the means of at least two determinations. Controls were without sugars or amino sugars.

Table 2. *The effect of N-acetylglucosamine and its analogues on chitin synthetase activity*

Substance*	Specific activity		
	Control†	With substance†	Percentage of control activity
GlcNAc	253	351	139
F-GlcNAc	253	312	123
I-GlcNAc	516	507	98
GalNAc	142	143	100
GlcNAc + F-GlcNAc	654	926	142
GlcNAc + I-GlcNAc	408	425	104
GlcNAc + GalNAc	392	603	154

* F-GlcNAc, *N*-fluoroacetyl-D-glucosamine (Kent & Dwek, 1971); I-GlcNAc, *N*-iodoacetyl-D-glucosamine (Kent, Ackers & White, 1970); GalNAc, *N*-acetylgalactosamine. All at 5 mM, with 1 mM-UDP-GlcNAc.

† Specific activities are expressed as nmol GlcNAc incorporated/min/mg protein. Values are the means of at least two determinations. Controls were without *N*-acetylglucosamine or its analogues.

Table 3. *The effect of adenine and uridine nucleotides on the activity of chitin synthetase from Coprinus cinereus*

Specific activity at:							
Nucleotide	Concn (mM)	Low substrate concn (0.1 mM-UDP-GlcNAc)			High substrate concn (1.0 mM-UDP-GlcNAc)		
		Control*	With nucleotide*	Percentage of control activity	Control*	With nucleotide*	Percentage of control activity
ATP	2.0	11.6	5.1	44	317	279	88
	0.5	11.6	9.0	76	325	325	100
ADP	2.0	11.6	5.6	48	317	274	86
	0.5	11.6	9.2	79	317	356	112
AMP	2.0	11.6	7.3	63	317	311	98
	0.5	11.6	9.0	76	317	338	107
UTP	2.0	12.7	0.7	6	284	143	50
	0.5	12.7	6.2	49	284	236	83
UDP	2.0	12.7	0.1	1	284	41	14
	0.5	12.7	2.2	17	284	159	56
UMP	2.0	12.7	1.1	9	284	151	53
	0.5	12.7	6.2	49	284	239	84

* Specific activities are expressed as nmol GlcNAc incorporated/min/mg protein. Most values are the means of at least two determinations. Controls were without nucleotides.

Table 4. *The effect of sugars, amino sugars and nucleotides on the activity of the nucleoside diphosphatase associated with chitin synthetase preparations*

Specific activity at:							
Substance	Concn (mM)	Low substrate concn (0.025 mM-UDP)			High substrate concn (0.425 mM-UDP)		
		Control	With substance	Percentage of control activity	Control	With substance	Percentage of control activity
Glucose	20	25.6	24.0	94	154	130	85
Glucosamine	5	28.5	26.3	92	154	140	92
Glucosamine-6-phosphate	5	28.5	19.6	69	154	135	88
N-acetylglucosamine	5	28.5	27.0	94	154	147	96
Diacetylchitobiose	2	28.5	27.5	97	143	128	96
ADP	2	25.6	10.4	41	145	82	57
AMP	2	25.6	21.1	82	145	148	102
UMP	2	25.6	19.6	77	145	140	97

* Specific activities are expressed as nmol UMP formed/min/mg protein. The chitin synthetase activity of the enzyme preparation giving an activity of 154 nmol UMP formed/min/mg protein was 481 nmol GlcNAc incorporated/min/mg protein under standard assay conditions. Most values are the means of at least two determinations. Controls were without sugars, amino sugars and nucleotides.

inhibitor of chitin synthetase, the UDPase action of this enzyme in our preparations clearly affects the observed kinetics by removing the product inhibitor, and we have investigated its activity. It has a requirement for magnesium ions and shows classical Michaelis-Menten enzyme kinetics, with a K_m of 0.35 mM-UDP.

The sugar and nucleotide effectors of chitin synthetase activity described above were

tested to see whether their activity could be explained by a primary action on the UDPase (Table 4). This only appears possible in the case of ADP and glucosamine-6-phosphate, where inhibition of the UDPase would result in UDP accumulating with a consequent inhibition of chitin synthetase.

DISCUSSION

The *Coprinus* chitin synthetase preparations do not show hyperbolic Michaelis–Menten kinetics, as graphs of velocity against UDP-*N*-acetylglucosamine concentration are sigmoidal and the corresponding Lineweaver–Burk double reciprocal plots are not linear. Enzyme preparations from *Mucor rouxii* (McMurrugh & Bartnicki-Garcia, 1971) and *Blastocladiella emersonii* (Camargo *et al.* 1967) show similar characteristics. Published results from other fungal preparations that do show linear Lineweaver–Burk plots appear to be linear only at higher substrate concentrations, usually in the presence of *N*-acetylglucosamine. Both of these factors favour an apparent linearity of this plot with our preparation, but any extrapolations are invalid as the enzyme preparation gives a Hill coefficient greater than one, so a double reciprocal plot will give a non-linear curve with a turning point at $1/[S] = 0$.

UDP-*N*-acetylglucosamine is thus an activator as well as a substrate of the *Coprinus* chitin synthetase. Although there are other possible explanations, this suggested that the chitin synthetase could be an allosteric enzyme (Monod, Changeux & Jacob, 1963). The binding of an effector to the enzyme is thought to bring about a conformational change (allosteric transition) of the protein, so that it more readily or less readily binds substrate. For allosteric enzymes, one of the substrates is also an allosteric effector, and hence if the *Coprinus* chitin synthetase is allosteric it must have more than one binding site for UDP-GlcNAc per enzyme molecule. The Hill number, n (calculated as the slope of the Hill plot or from the adapted Fajsz and Endrenyi plot) is a measure of the number of substrate-binding sites and the degree of interaction between them (Atkinson *et al.* 1965). If there is a strong interaction, the value of n will approximate to the number of binding sites, but weaker interactions or a reduction of the number of sub-units per molecule will reduce the number towards 1.0.

By both methods of calculation, the Hill number for *Coprinus* chitin synthetase was 3.6 to 4.0 at UDP-GlcNAc concentrations below 0.1 mM and 1.7 at higher concentrations. We interpret this as indicating that the enzyme has four UDP-GlcNAc binding sites. Increasing the UDP-GlcNAc concentration would result in a conformational change of the enzyme after the binding of the first molecule, to favour the binding of more molecules, with a consequent increase in enzyme activity. The reduction in value of the Hill number at higher UDP-GlcNAc concentrations may reflect a reduced interaction between UDP-GlcNAc binding sites.

Whatever the mechanism, the fact that the enzyme has little activity at low UDP-GlcNAc concentrations will ensure that it is only active when there is enough substrate present in its vicinity for long chains of chitin to be produced.

As with other chitin synthetase systems, the enzyme from *Coprinus* is activated by *N*-acetylglucosamine. This substance is not incorporated into the enzyme product (Gooday & de Rousset-Hall, 1975). Both *N*-acetylglucosamine and its disaccharide, diacetylchitobiose, activate only at low substrate concentrations (see Fig. 1). It is suggested that they are positive allosteric effectors, binding to the enzyme to cause a conformational change so that it more readily binds UDP-GlcNAc. As these effectors do not activate at high substrate concentrations, this conformational change may be similar to that produced by UDP-GlcNAc.

However, glucose activates at all UDP-GlcNAc concentrations and so presumably has a different action.

The increase in chitin content during the development of the *Coprinus* stipe involves intussusception of new chitin microfibrils among pre-existing ones (Gooday, 1972*a, b*, 1973). This must involve the co-ordinate action of chitinase and chitin synthetase: breaking down the older microfibrils to insert the new ones. The activation of the anabolic enzyme, chitin synthetase, by *N*-acetylglucosamine and diacetylchitobiose (the products of the catabolic enzyme, chitinase) may play an important role in controlling this development sequence.

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