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IDENTIFICATION OF GTP AS A PHYSIOLOGICALLY RELEVANT INHIBITOR OF *Escherichia coli* ADP-GLUCOSE SYNTHETASE

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SUMMARY. We show that physiological concentrations of GTP can significantly inhibit wild-type *Escherichia coli* ADP-glucose synthetase (the rate-limiting enzyme of bacterial glycogen synthesis) and that mutant-strain enzymes known to show less inhibition by physiological AMP levels also show less inhibition by physiological levels of GTP. This decreased inhibition by both AMP and GTP can almost totally account for the higher cellular rates of glycogen synthesis observed in the mutant strains. In addition, in metabolic conditions where we have shown that cellular glycogen synthesis increases, cellular GTP levels are known to decrease. Thus, we conclude that GTP inhibition is physiologically relevant.

ADP-glucose synthetase (EC 2.7.7.27), which catalyzes the reaction ATP + glucose-1-P → ADP-glucose + PP_i, is the rate-limiting enzyme of bacterial glycogen synthesis (1). Previous work from our laboratory has attempted to correlate measured changes in the cellular rate of glycogen synthesis in *Escherichia coli* with estimated changes in the *in vivo* rate of ADP-glucose synthetase. One of the consequences of that work was our hypothesis that a previously undetected inhibitor of that enzyme is present in *E. coli* during growth on glucose (2). We show here for the first time that GTP can inhibit *E. coli* ADP-glucose synthetase and that significant inhibition can be obtained with the concentration of GTP known to occur in glucose-grown *E. coli*. Using mutant strains that possess an ADP-glucose synthetase that we show to be less inhibited by the physiological concentration of GTP than is the wild-type enzyme, we provide evidence that the inhibition by GTP is physiologically relevant. We also provide evidence that inorganic pyrophosphate, which was suggested by others (3) as a candidate for our hypothetical inhibitor, is not a physiologically relevant inhibitor of ADP-glucose synthetase.

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METHODS. E. coli B, SG5 and CL1136 (4,5) were provided by Dr. Jack Preiss (University of California, Davis). E. coli W4597(K) is a UDP-glucose synthetase-deficient derivative of E. coli K-12 (6). Cells were grown at 32° as previously described (6,7). Glycogen and protein were measured and rates of glycogen synthesis were calculated as described previously (7). Nucleotides and 6-azauracil were obtained from Sigma Chemical. ADP-glucose synthetase activity was measured in crude extracts, prepared as previously described (8). Total enzyme activity was determined as previously described (saturating concentrations of substrates and fructose-1,6-P_2, energy charge 1.0)(8), but an incubation temperature of 32° was used here.

RESULTS. Treatment of glucose-grown E. coli with agents that alter de novo nucleotide synthesis appeared to cause changes in the hypothetical inhibitor of ADP-glucose synthetase (2). Therefore, we tested various ribonucleotides at the concentrations found in glucose-grown E. coli (9-13) (or in cases where E. coli data were not available, at concentrations found in glucose-grown Salmonella typhimurium (14,15)) for inhibition of the enzyme from E. coli B (Table 1). (Data for AMP and ADP are not shown for two reasons. First, it

TABLE 1
Effect of physiological concentrations of ribonucleotide phosphates on activity of ADP-glucose synthetase of E. coli B

Addition	Concentration	ADP-Glucose synthetase	Activity remaining
	mM	μmol/g of protein/h	%
None	—	645	100
GTP	1.0	375	58
GDP	0.1	643	100
GMP	0.1	617	96
CTP	0.5	548	85
CDP	0.1	567	88
CMP	0.1	576	89
ZTP[a]	0.1	646	100
ZDP	0.1	565	88
ZMP	0.1	587	91
UMP	0.1	644	100
IMP	0.1	662	103
XMP	0.1	600	93
None[b]	—	2010	100
UTP	0.8	1600	80
UDP	0.1	1830	91
UMP	0.1	2010	100
GTP	1.0	1090	54

[a] The symbol Z represents AICA-riboside (5-aminoimidazole-4-carboxamide riboside)(14,15).

[b] UTP and UDP inhibition could not be measured with an E. coli B crude extract because it contains UDP-glucose synthetase activity and an activity that converts UDP to UTP. Therefore, UTP and UDP inhibition was tested with a crude extract of the UDP-glucose synthetase-deficient strain E. coli W4597(K). The ADP-glucose synthetase from this strain shows about the same degree of inhibition by GTP as the enzyme from E. coli B.

TABLE 2

Maximum velocity (V) of ADP-glucose synthetase, and apparent maximum velocity (V_g) in the presence of 1 mM GTP

E. coli strain	V	V_g	Activity remaining[a]
	μmol/g of protein/h		%
B	640	370	58
SG5	797	599	75
CL1136	638	635	100

[a] $(V_g/V) \times 100$

is already known that, at physiological concentrations, AMP does but ADP does not inhibit the purified E. coli B enzyme (5,16). Second, these points are not clearly shown with a crude extract such as we used here, because, as we showed previously (8), the high adenylate kinase activity of the preparation causes rapid interconversion of AMP and ADP.) Of the ribonucleotides tested, the physiological concentration of GTP found in glucose-grown E. coli B, 1 mM (9-11), showed the most striking inhibition, approximately 40% (Table 1).

We next tested the effect of 1 mM GTP on the activity of the ADP-glucose synthetase from two different mutant derivatives of E. coli B that are known to possess an altered form of the enzyme. The enzyme from the mutant strain SG5 showed less inhibition than the wild-type enzyme, and the enzyme from the mutant strain CL1136 was totally free from inhibition by 1 mM GTP (Table 2). It is already well known that with 80 μM AMP, the concentration found in glucose-grown E. coli (17), the SG5 enzyme is less inhibited than the B enzyme and the CL1136 enzyme is not inhibited at all (4,5) (Table 3). However, we show here that the decreased inhibition by this physiological level of AMP can account for only a small portion of the higher rates of glycogen synthesis that have been observed by Preiss and co-workers in glucose-grown E. coli SG5 and CL1136 relative to glucose-grown E. coli B (4,5). In contrast, the combined decrease in inhibition by physiological levels of AMP and GTP can account for a large portion of the higher rates in the mutant strains.

The ATP concentration, 2.3 mM, and the fructose-1,6-P_2 concentration, 3 mM, observed in E. coli growing on glucose (18) are saturating for the

TABLE 3

Fraction of ADP-glucose synthetase maximal activity (V) remaining in the presence of 1 mM GTP (V_g/V), of 80 μM AMP (V_a/V), or of both ($(V_g/V) \times (V_a/V)$)

E. coli strain	V_g/V	V_a/V	$(V_g/V) \times (V_a/V)$	$\frac{(V_g/V)_M (V_a/V)_M}{(V_g/V)_B (V_a/V)_B}$ [a]
B	0.58	0.60	0.35	—
SG5	0.75	0.76	0.57	1.6
CL1136	1.00	1.00	1.00	2.9

The values of V_g/V were obtained from the data in Table 2. The values of V_a/V were determined by Preiss and co-workers for the purified enzymes (4,5) by measuring the activity at adenylate energy charge 1.0 (V, AMP absent) and at the physiological energy charge 0.85 (V_a, 80 μM AMP present).

[a] The subscript M refers to the appropriate mutant strain, SG5 or CL1136; the subscript B denotes strain B. These ratios are equal to the calculated relative rates of ADP-glucose synthetase *in vivo* v_{SG5}/v_B and v_{CL1136}/v_B, respectively (see text).

E. coli B, SG5 and CL1136 enzymes (4,5). Thus, in this physiological condition the rate equation for ADP-glucose synthetase (8) reduces to

$$v = \frac{[(V_g/V)\ (V_a/V)]\ V}{1 + (S_{0.5}/\text{Glc-1-P})} \qquad [1]$$

Multiplication of the maximal activity (V) by the product $(V_g/V)\ (V_a/V)$ (Table 3) gives the maximum velocity in the presence of physiological concentrations of the inhibitors GTP and AMP. The value of V (total enzyme activity in crude extracts) is the same for glucose-grown cultures of B, SG5 and CL1136 when the growth conditions of Preiss and co-workers are used (4,5), and it seems reasonable to assume that in these conditions the cellular concentration of glucose-1-P is the same for all three strains (7); the value of $S_{0.5}$ is the same for the B, SG5 and CL1136 enzymes (4,5). Consequently, in Equation 1 only the values of the product $(V_g/V)\ (V_a/V)$ are different for each of the strains (Table 3), which reflects their differences in inhibition by physiological AMP plus GTP. Therefore, when Equation 1 is used to calculate v_{SG5}/v_B and v_{CL1136}/v_B, the expected relative rates of ADP-glucose synthetase *in vivo*, the values obtained are equal to the values of the ratios in the last column of Table 3, which are approximately 1.5 and 3.0, respec-

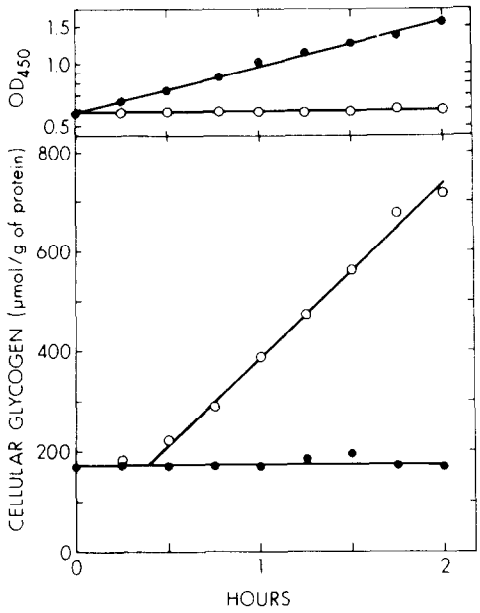

Fig. 1. Effect of 6-azauracil on growth and cellular glycogen accumulation in E. coli W4597(K): no addition (●); azauracil, 150 μg/l final concentration (o). Glycogen is expressed as glycogen glucose. The cellular rate of glycogen synthesis in the untreated control culture is 86 μmol/g of protein/h and the cellular rate in the azauracil-treated culture is 350. The rate in the control culture is obtained from the product of the growth constant [(ln 2)/generation time] and the steady-state cellular level of glycogen (7), or [(ln 2)/1.4 h][173 μmol/g of protein]. The cellular rate in the treated culture is equal to the slope of the line that reflects the linear accumulation of cellular glycogen (7).

tively. These values account for 75% of the 2- and 4-fold higher respective rates of cellular glycogen synthesis that have been observed by Preiss and co-workers (4,5) in glucose-grown E. coli SG5 and CL1136 relative to glucose-grown E. coli B. If only the decreased inhibition by AMP had been considered, only 1.3-fold and 1.7-fold higher rates would have been predicted (calculate $(V_a/V)_{SG5}/(V_a/V)_B$ and $(V_a/V)_{CL1136}/(V_a/V)_B$ from the values in Table 3).

Since we proposed the existence of the hypothetical inhibitor (2), Preiss and Greenberg (3) suggested that inorganic pyrophosphate may fulfill the role of the inhibitor. However, we show here that treatment of glucose-grown E. coli with 6-azauracil causes a large increase in the cellular rate of glycogen synthesis (Fig. 1) despite the fact that such treatment is known to increase the cellular level of inorganic pyrophosphate (19) to a level that completely inhibits ADP-glucose synthetase in vitro (3).

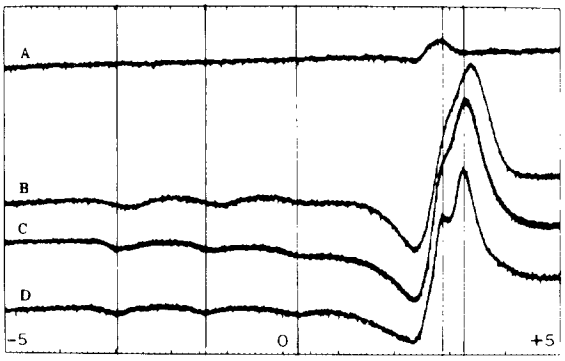

Figure 4 : The low temperature EPR spectra of copper. All spectra were recorded at liquid nitrogen with modulation amplitude of 10 Gauss. All samples contained copper sulfate (1mM) in phosphate buffer (5mM, pH7.4)
A. No additions
B. Addition of AChE (10mg/ml)
C. Addition of AChE (10mg/ml) together with decomposed divicine (5mM)
D. Addition of decomposed divicine (5mM).

decomposed pyrimidine provided protection to the enzyme, possibly by chelating of the copper ions.

Figure 4 shows the low temperature EPR spectra of copper(II) in various systems. It can be seen that copper formed a complex with the decomposed divicine (trace D), and with AChE (trace B). In the presence of both AChE and decomposed divicine (trace C), the spectral features were similar to those in trace D, indicating that now the copper was bound to the decomposed pyrimidine.

DISCUSSION

We have used the inactivation of a protein, purified or membrane-bound acetylcholine esterase, as a simplified model for the mechanism of biological damage in the hemolytic crisis in favism. The results show that loss of enzymatic function is dependent on the presence of a favism-inducing agent (isouramil or divicine) together with copper or iron (not shown) ions. Chelating agents totally protected against the deleterious effects. Enzymatic inactivation was also dependent on oxygen,

basal, essentially non-inhibitory level (Table 1), to a higher level (15,23) that we have shown to severely inhibit ADP-glucose synthetase in vitro (2). Because of these offsetting effects one would expect that treatment of E. coli with decoyinine, also an inhibitor of GMP synthetase, would have little effect on the cellular rate of glycogen synthesis, exactly what we observed previously (2). Similarly, hadacidin decreases GTP but also increases ZMP and IMP (23) to inhibitory levels (2); as expected the cellular rate of glycogen synthesis of E. coli shows little change with hadacidin treatment (2). Treatment with azaserine, which should decrease GTP, ZMP and XMP, shows the expected stimulation of the cellular rate of glycogen synthesis (2).

In summary, many observations support, and we are not aware of any observations in conflict with, our conclusion that the cellular level of GTP plays a role in regulating glycogen synthesis in the intact E. coli cell. In addition, the newly observed inhibition by GTP may at last provide the mechanism for the old observation that the cellular rate of glycogen synthesis increases as the growth rate decreases (24), because the cellular concentration of GTP decreases as the growth rate decreases (12).

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