Involvement of Arginine Residues in the Allosteric Activation of *Escherichia coli* ADP-glucose Synthetase†

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ABSTRACT: Inactivation of *Escherichia coli* ADP-glucose synthetase (EC 2.7.2.27) by the arginine-specific reagents cyclohexanedione and phenylglyoxal resulted primarily from interference with normal allosteric activation. Partial modification by phenylglyoxal resulted in a lessened ability of fructose 1,6-bisphosphate (fructose-P_2) to stimulate and of 5′-AMP (5′-adenylate) to inhibit enzymic activity. The apparent affinity for fructose-P_2 and the V_{max} at saturating fructose-P_2 concentrations were decreased by the arginine modification. Fructose-P_2, 5′-adenylate, and several other allosteric effectors were able to partially protect the enzyme from inactivation. However, catalytic activity was not decreased by arginine modification under conditions where the enzyme was assayed in the absence of fructose-P_2. The two arginine-modifying reagents differed markedly in their reactivity with the enzyme. Cyclohexanedione inactivated the enzyme quite slowly and eventually reacted with at least 14 of the 32 arginines present per subunit. Phenylglyoxal was some 50-fold more effective in inactivation, but it modified only one arginine residue per subunit.

ADP-glucose synthetase (EC 2.7.7.27) catalyzes the rate-limiting step in bacterial glycogen biosynthesis, the formation of the glucosyl donor ADP-glucose (Preiss, 1969, 1973, 1978; Preiss & Walsh, 1981). Recent studies on the purified enzyme from *Escherichia coli* B strain AC70R1 have indicated that it is a tetramer with a molecular weight of about 2×10^5 and is composed of identical subunits (Haugen et al., 1976a). The enzyme activity is allosterically regulated by glycolytic intermediates, notably fructose 1,6-bisphosphate (fructose-P_2)[1] (Preiss et al., 1966), and by the overall energy charge (Shen & Atkinson, 1970). A survey of activators suggested that the region of the *E. coli* enzyme around their common binding site must contain at least two residues with cationic side chains (Preiss, 1972). Pyridoxal phosphate was recently shown to react with a lysine in the vicinity of the allosteric activator binding site (Haugen et al., 1976b; Parsons & Preiss, 1978a), and a peptide containing pyridoxylated lysine was isolated and partially sequenced (Parsons & Preiss, 1978b). Chemical modification studies with trinitrobenzenesulfonate, another reagent specific for ϵ-amino groups, revealed additional lysine residues(s) essential to allosteric regulation which were in the same region (Carlson & Preiss, 1981). We present evidence here that the region critical to allosteric activation also contains an essential arginine residue whose guanidinium side chain may be involved in the binding of allosteric effectors.

Experimental Procedures

Reagents. Cyclohexanedione (CHD) and phenylglyoxal (PG) were purchased from the Aldrich Chemical Co. [^{14}C]-CHD (3.50 mCi/mmol) was from New England Nuclear. [^{14}C]Acetophenone was a product of International Chemical & Nuclear. Hexanediol-P_2 was synthesized as described by Hartman & Barker (1965). Spectrapor 3 dialysis tubing was from Spectrum Medical Industries. Fluorescamine was purchased from Roche Diagnostics. All other reagents were of the highest commercial grade available.

Isolation of Enzyme. The homogeneous ADP-glucose synthetase used in this study was from strain AC70R1 of *E. coli* B, prepared as described (Haugen et al., 1976a).

Protein was assayed by the microscale modification (Layne, 1957) of the method of Lowry. The concentration was also measured by the absorbance at 280 nm by using the relation $A^{1cm}_{280nm} = 1.0$ mg/mL (Haugen et al., 1976b).

Synthesis of [^{14}C]Phenylglyoxal. [^{14}C]PG was synthesized from [^{14}C]acetophenone by the method of Riley & Gray (1947). The product was repeatedly crystallized from hot water; the final yield was 49%, and the specific radioactivity was 0.105 mCi/mmol. Identity was confirmed by the production of α-(dinitrophenyl)di[^{14}C]PG–arginine by the method of Takahashi (1968).

ADP-glucose Synthetase Assays. (A) Synthesis Direction. Assay for the synthesis of ADP-[^{14}C]glucose from [^{14}C]-glucose-1-P and ATP was routinely used as described (Ghosh & Preiss, 1966). The reaction mixture (0.20 mL) contained 20 μmol of Hepes, pH 7.0, 100 μg of bovine serum albumin, 0.1 μmol of [^{14}C]glucose-1-P [$(5–10) \times 10^5$ cpm/μmol], 0.3 μmol of ATP, 1.0 μmol of $MgCl_2$, 0.5 μg of yeast inorganic pyrophosphatase, 0.3 μmol of fructose-P_2, and enzyme.

(B) Pyrophosphorolysis Direction. Assay for the formation of [^{32}P]ATP from ADP-glucose and $^{32}PP_i$ was routinely used as described (Shen & Preiss, 1964). The reaction mixture contained (in 0.25 mL) 10 μmol of Tris-HCl, pH 8.5, 100 μg of bovine serum albumin, 2.0 μmol of $MgCl_2$, 0.2 μmol of ADP-glucose, 0.5 μmol of $^{32}PP_i$ [$(5–30) \times 10^6$ cpm/μmol], 0.3 μmol of fructose-P_2, and enzyme. All enzyme assays were done at 37 °C, initiated by the addition of enzyme, and measured at a reaction rate proportional to time and enzyme concentration. For assay in either direction, a unit of activity is defined as that amount of enzyme which catalyzed the formation of 1 μmol of product per min.

Treatment of Data To Obtain Kinetic Constants. V_{max} was determined from double-reciprocal plots of reaction velocity vs. effector concentration. Hill plots were used for determination of the following parameters: $S_{0.5}$, $A_{0.5}$, or $I_{0.5}$, which correspond to the concentration of substrate, activator, or inhibitor required for half-maximal velocity, activation, or

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[1] Abbreviations: [^{14}C]CHD, 1,2-[1-^{14}C]cyclohexanedione; [^{14}C]PG, [1-^{14}C]phenylglyoxal; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; fructose-P_2, fructose 1,6-bisphosphate; hexanediol-P_2, hexanediol 1,6-bisphosphate; 5′-AMP, 5′-adenylate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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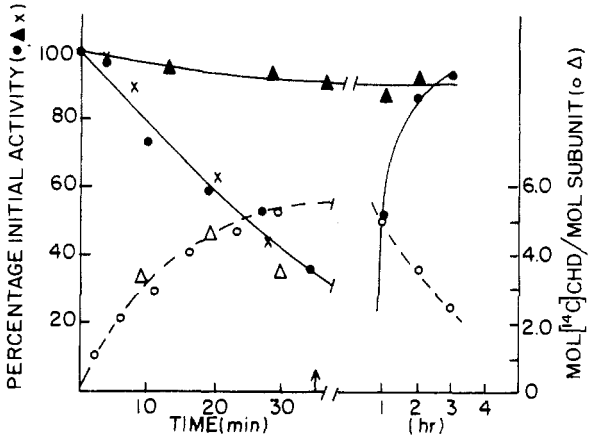

FIGURE 1: Reversible inactivation of enzyme by incorporation of [^{14}C]CHD. Enzyme (20 μm subunits) was incubated at 25 °C with 10 mM [^{14}C]CHD in the presence (●, O) or absence (×, Δ) of 50 mM $Na_2B_4O_7$. The control (▲) was incubated with $Na_2B_4O_7$ but without CHD. Aliquots were removed at different time points for measurement of incorporated radioactivity (open symbols) or fructose-P_2-stimulated enzyme activity (closed symbols). After 35 min (arrow), 0.2 m NH_2OH was added, and the incubation was continued at 37 °C.

inhibition, respectively, and $\bar{n}$, the Hill interaction coefficient (Taketa & Pogell, 1965; Changeux, 1963; Atkinson et al., 1965).

Reaction with Cyclohexanedione. Enzyme was dialyzed against 0.1 M Hepes buffer, pH 7.0, containing 0.5 mM dithioerythritol. It was treated at 25 °C in the dark under N_2 (Patthy & Smith, 1975a) with a fresh solution of CHD in 0.05 M $NaHCO_3$ buffer, pH 8.1, containing 0.05 M $Na_2B_4O_7$. Samples for assay of enzyme activity were removed at time points and diluted 2000-fold into cold 0.05 M $Na_2B_4O_7$, pH 8.9. For estimation of incorporated radioactivity, reaction aliquots were diluted 5-fold and immediately dialyzed in the dark against cold 0.2 M K_2HPO_4 buffer, pH 8.9, containing 0.25 mM EDTA, 0.5 mM dithioerythritol, and 0.05 M $Na_2B_4O_7$. The higher ionic strength was necessary to minimize interference due to noncovalently bound radioactivity.

For amino acid analysis, [^{14}C]CHD–enzyme was dialyzed extensively in the dark against 5% acetic acid. Norleucine was added as an internal standard, and samples were lyophilized and then hydrolyzed at 110 °C for 24 h in constant-boiling 6 N HCl plus 5% mercaptoacetic acid in evacuated sealed vials. The hydrolyzed samples were dried and dissolved in citrate amino acid analysis buffer to run on the Durrum D-500 analyzer.

Reaction with Phenylglyoxal. Enzyme in 0.05 M K_2HPO_4 buffer, pH 7.5, containing 0.25 mM EDTA, was treated with PG in 0.05 M Mops buffer, pH 8.0, at 25 °C. Aliquots for assay were diluted 5000-fold into cold Mops buffer containing 1 mM arginine to stop the reaction. Reaction samples to be measured for [^{14}C]PG incorporation were immediately filtered on 1.3-cm^3 columns of Sephadex G-25 in 0.05 M Mops buffer, pH 8.0, to remove unincorporated radioactivity. This method was used when only a few samples were involved. Alternatively, reaction samples were treated immediately with sodium borohydride in a 15-fold molar excess of PG to stop the reaction by reducing PG to its glycol and then extensively dialyzed against 0.1 M $NaHCO_3$ buffer, pH 8.5, containing 0.2 M KCl until the first buffer change.

Results

Inactivation by Cyclohexanedione. ADP-glucose synthetase assayed in the direction of synthesis (see Experimental Procedures) was rapidly inactivated by 10 mM [^{14}C]CHD in $NaHCO_3$ buffer at pH 8.1 and 25 °C (Figure 1). Enzyme-associated radioactivity which was stable to dialysis appeared and increased as enzyme activity decreased (Figure 1). In the incubation conditions of Figure 1, the enzyme was completely stable in the absence of [^{14}C]CHD. The presence of sodium borate alone had no effect on enzyme activity and was not necessary for inactivation by CHD but did help stabilize the radioactive label during dialysis. It was included in subsequent experiments.

Table I: Arginine Composition of [^{14}C]CHD-Modified Enzyme[a]

sample	mol of [^{14}C]CHD/ mol of subunit	mol of Arg/ mol of subunit
unmodified	0	32.1 ± 1.9[b]
modified enzyme	0.7	29.6
	2.8	27.7
	4.3	21.6

[a] Enzyme (20 μM) was incubated with 2, 10, or 20 mM [^{14}C]-CHD for 30 min, then assayed for incorporated radioactivity, and prepared for amino acid analysis as outlined under Experimental Procedures. [b] Average of determinations of Haugen et al. (1976a), Carlson et al. (1976), and Kappel & Preiss (1981).

Modification by CHD was reversible by hydroxylamine at 37 °C (Figure 1). Slow but complete reactivation of the enzyme accompanied loss of enzyme-bound radioactivity.

Stoichiometry and Site of CHD Modification. A large number of amino acid residues were reacting with [^{14}C]CHD under these conditions, if each derivative was formed by one molecule each of CHD and borate (Patthy & Smith, 1975a). The data of Figure 1 indicated that more than 6 mol of CHD/mol of enzyme subunit would accompany complete inactivation. In a subsequent experiment, up to 14 mol of [^{14}C]CHD/mol of subunit was actually incorporated after 30 min with 20 mM [^{14}C]CHD.

ADP-glucose synthetase contains some 32 arginines per 50 000 molecular weight subunit, offering numerous sites for modification by cyclohexanedione. Amino acid analysis of modified enzyme showed that arginines were involved in the reaction with CHD (Table I). This approach was feasible since Patthy & Smith (1975a) have shown that acid hydrolysis of the derivative does not regenerate arginine. The large number of arginines in ADP-glucose synthetase lent some margin of error in their estimation by amino acid analysis. Nevertheless, a significant decrease in unmodified arginines accompanied increased incorporation of [^{14}C]CHD.

Kinetics of CHD Modifications. The chemical evidence indicated that modification by CHD was extensive and apparently nonspecific. However, several kinetic experiments demonstrated a localized effect upon catalysis and regulation of the enzyme.

The reaction order for the inactivation process was determined from the half-time for inactivation at different concentrations of nonradioactive CHD. A log–log plot of these data (not shown) gave a minimum value for the reaction order, as described by Levy et al. (1963) and others (Scrutton & Utter, 1965; Keech & Farrant, 1968; Hollenberg et al., 1971). The value of 0.93 for the slope (n) suggested that the reaction of only one CHD with each enzyme subunit (i.e., one Arg per subunit) was sufficient for the initial inactivation.

In a second experiment, partially modified enzyme preparations were also assayed without fructose-P_2, the allosteric activator which is usually added. There was a significant difference in the effect of modification upon the activities with or without fructose-P_2 (Figure 2). Only the fructose-P_2-stimulated activity was rapidly lost during the initial stages of CHD modification as shown by the sharp decrease in the

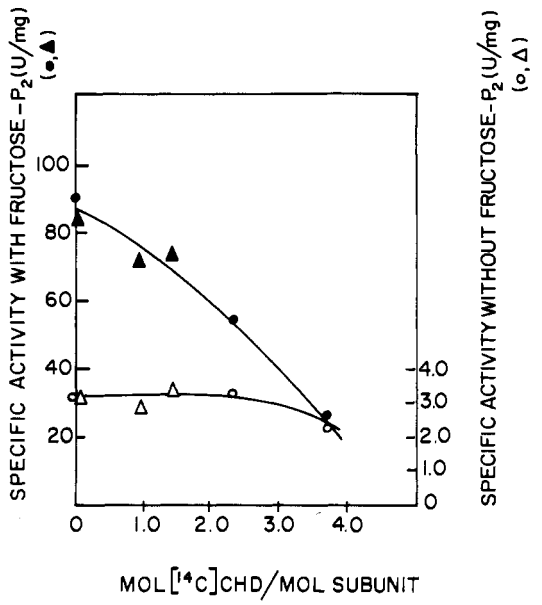

FIGURE 2: Effect of CHD upon fructose-P_2-stimulated activity. Enzyme (20 μM) was modified to varying extents by incubation for 30 min with 2.5–10 mM [^{14}C]CHD in the presence of 30 mM $Na_2B_4O_7$, 2 mM ADP-glucose, and 20 mM $MgCl_2$. Activity was assayed in the presence (●, ▲) or absence (○, △) of the allosteric activator fructose-P_2. The triangles and circles represent data from two experiments.

+fructose-P_2/−fructose-P_2 activity ratio. The lower level of activity measured in the absence of fructose-P_2 was unchanged until nearly 3 mol of CHD had been incorporated per mol of subunit. Enzyme prepared for these studies was modified in the presence of substrate ADP-glucose so that the catalytic site would be largely protected from reaction with CHD. Therefore, the initial loss of activated activity specifically implicated the activator site as a target of modification.

Other experiments showed that in fact both substrate and activator could protect enzyme activity during reaction with CHD, an observation consistent with the evident lack of modification specificity. For example, in the presence of 2 mM ADP-glucose (plus 20 mM $MgCl_2$) or 2 mM fructose-P_2, or both, the enzyme retained 52%, 47%, or 89%, respectively, of its initial activity after 30 min, compared to only 24% when unprotected (data not shown). The incorporation of [^{14}C]CHD was still extensive however.

In summation, the studies with cyclohexanedione indicated that one effect was specific interference with allosteric activation of the enzyme, that arginine residues were involved, and that initial loss of fructose-P_2-stimulated activity could result from modification of as few as one of these residues.

Inactivation by Phenylglyoxal. The modification of ADP-glucose synthetase with phenylgloxal was also studied, since the different chemical properties of the reagent, yielding a different structure of the arginine derivative, might give it a specificity manifest in different rate, extent, and effect of modification, compared to CHD. This proved to be true.

PG rapidly inactivated ADP-glucose synthetase at pH 8.0 in Mops buffer, when assayed in either the forward (synthesis) or the reverse (pyrophosphorolysis) direction (Figure 3A). The rate of inactivation of synthesis activity was 2–3-fold greater than the pyrophosphorolysis activity, at a given concentration of PG. In both assays, the loss of more than 90% of the activity followed pseudo-first-order kinetics, giving a linear plot of activity vs. time on a semilogarithmic graph. Furthermore, the half-life of inactivation was proportional to PG concentrations up to 80-fold excess over the concentration of enzyme subunits. Both enzyme activities were completely stable for more than 60 min in the absence of PG.

The inactivation by PG was much more rapid than by CHD. An 8-fold molar excess of PG over enzyme subunit concentration led to half-inactivation of synthesis in 24 min (Figure 3) while a comparable inactivation required a 500-fold molar excess of CHD (Figure 1).

When the order for the inactivaton process was determined, the values $n = 0.90$ for synthesis and $n = 1.05$ for pyro-

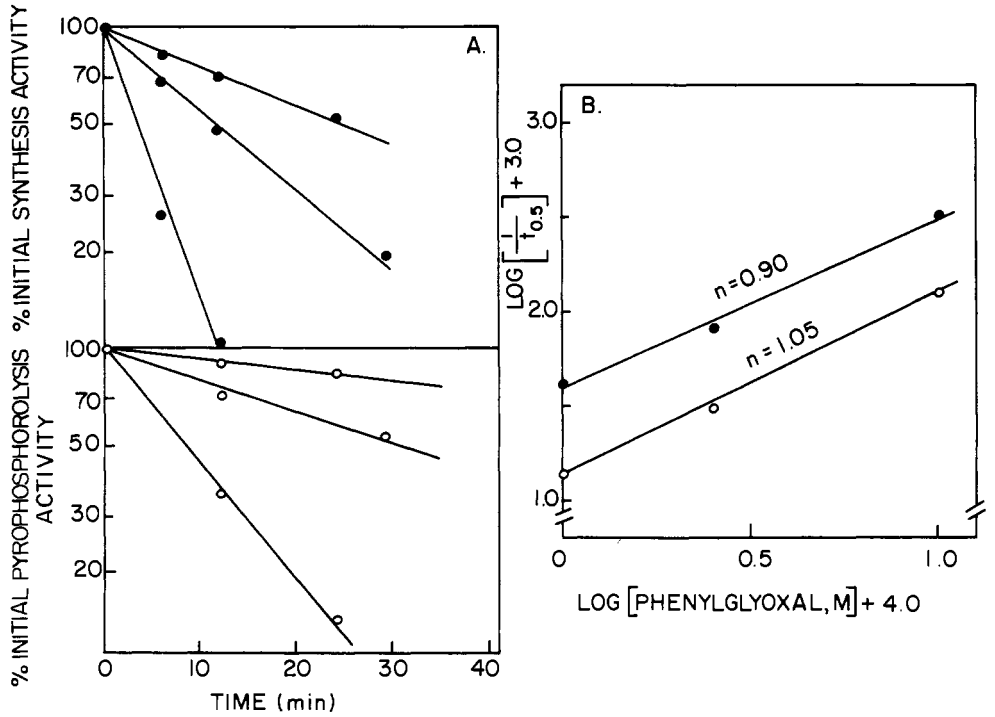

FIGURE 3: Kinetics of enzyme inactivation by PG. (A) The loss with time of synthesis (●) and pyrophosphorolysis (○) activities of 12 μM enzyme incubated with the following concentrations of PG: 0.1, 0.25, and 1.0 mM. Both activities were assayed in the presence of fructose-P_2. (B) Determination of the order of the inactivation reaction for synthesis (●) and pyrophosphorolysis (○).

Table II: Effect of Modification upon Response to Allosteric Effectors[a]

mol of [^{14}C]PG/ mol of subunit	fructose-P_2			5′-adenylate		
	V_{max} (%)	$A_{0.5}$ (mM)	$\bar{n}$	$V_{initial}$ (%)	$I_{0.5}$ (mM)	$\bar{n}$
0	100	0.10	1.4	100	0.10	1.6
0.12				59	0.09	1.5
0.43	27	0.13	1.5	20	0.11	1.4
1.1	18	0.20	1.3	9	0.12	1.1

[a] After incubation of 12 μM enzyme with 1.0 mM [^{14}C]PG for varying times, the activation by fructose-P_2 was measured in the synthesis direction. The initial specific activity was 94.5 units/mg. In a separate similar experiment, the inhibition by 5′-adenylate in the presence of fructose-P_2 was examined.

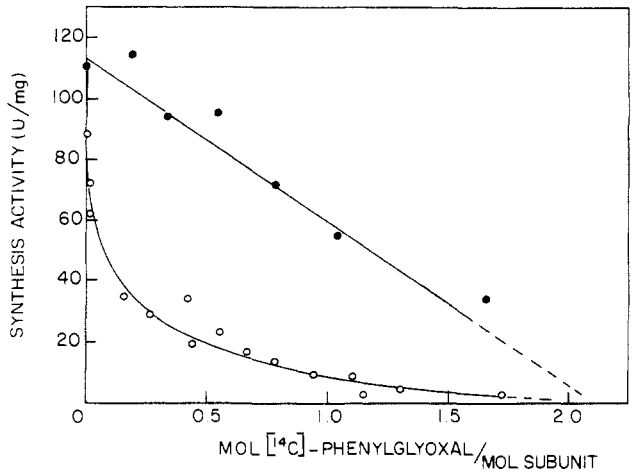

FIGURE 4: Stoichiometry of PG modification. In two separate experiments, 50 μM enzyme was incubated for different times with 6.0 mM [^{14}C]PG in $NaHCO_3$ buffer in the presence of substrate (2 mM ADP-glucose plus 20 mM $MgCl_2$) (●) or with 1.0 mM [^{14}C]PG in Mops buffer alone (○). Aliquots were assayed for incorporated radioactivity and fructose-P_2-stimulated synthesis activity.

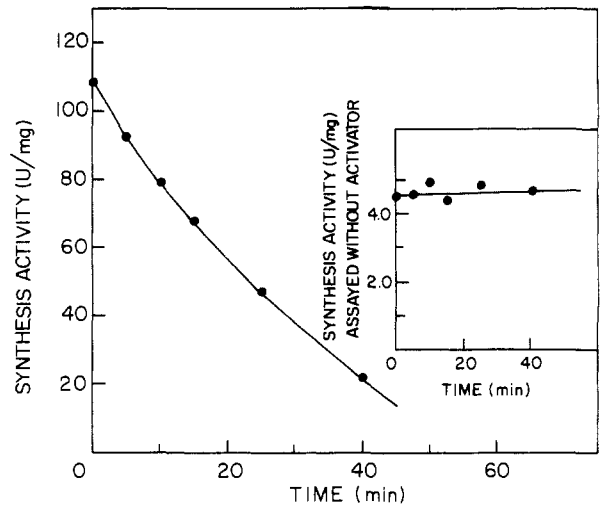

FIGURE 5: Effect of PG upon fructose-P_2-stimulated activity. Enzyme (50 μM) was incubated in the presence of substrate with 0.6 mM PG. Activity was assayed in fructose-P_2-stimulated synthesis. Inset: Enzyme activity assayed in the absence of fructose-P_2.

phosphorolysis suggested that loss of either activity resulted from reaction of as few as one molecule of PG per enzyme subunit (Figure 3B).

Stoichiometry of Modification. The stoichiometry of the covalent modification by PG of the enzyme was examined directly by measuring [^{14}C]PG incorporation during inactivation. Two moles of [^{14}C]PG per mole of subunit accompanied the complete loss of synthesis activity (Figure 4). If two molecules of PG react per arginine residue (Takahashi, 1968), then modification of one residue was sufficient for inactivation here.

The inactivation of synthesis activity was markedly altered when the substrate ADP-glucose was present during inactivation (Figure 4). The net amount of label which accompanied complete inactivation was the same, but the progressive loss of activity up to this point was much more directly proportional to modification than in the absence of substrate. Therefore, subsequent modification experiments were done with substrate present.

In light of the CHD effect upon enzyme stimulation by its activator fructose-P_2, PG-modified enzyme was examined in the same way to see if the limited extent of modification evident with PG was also directed at the allosteric properties. Fructose-P_2-stimulated activity was rapidly lost upon modification, but the low unstimulated enzyme activity was unaffected (Figure 5). The modification was comparable to that shown in Figure 4: incorporation of 1.6 mol of [^{14}C]-PG/mol of subunit accompanied 80% loss of stimulated synthesis activity.

Saturation Kinetics for Allosteric Effectors and Substrates. It was evident that PG incorporation affected normal allosteric regulation. This was further substantiated by kinetic evidence for both fructose-P_2 and the allosteric inhibitor 5′-AMP.

The saturation by fructose-P_2 was examined for enzyme modified to varying extents. As the extent of modification increased, the maximum velocity for synthesis activity attainable with saturating concentrations of fructose-P_2 was reduced (Table II). Furthermore, the concentration of fructose-P_2 required for half-maximal activation increased 2-fold. The Hill coefficient, $\bar{n}$, denoting the strength of the interaction between subunits, was not significantly changed.

Modification exerted a complex effect upon enzyme inhibition by 5′-AMP. As assayed in the presence of fructose-P_2 (Table II), the inhibition of modified enzyme preparations gave essentially the same $I_{0.5}$ and $\bar{n}$ values as those of unmodified enzyme. However, when activity was assayed in the absence of fructose-P_2 (Figure 6), the enzyme was progressively less inhibited by 5′-AMP even at high concentrations.

The substrate saturation kinetics of unmodified and half-inactivated enzyme preparations were also examined (data not shown). In the direction of synthesis and pyrophosphorolysis, the V_{max} for each decreased about 2-fold. The $S_{0.5}$ value for glucose-1-P was unchanged, but for ATP, it increased about 50% to 0.65 mM. The Hill slope value, $\bar{n}$, for ATP decreased from 2.0 to 1.5. The $S_{0.5}$ for ADP-glucose increased nearly 4-fold, and its Hill slope, $\bar{n}$, decreased from 1.9 to 1.15. For pyrophosphate, neither the $S_{0.5}$ value nor the Hill slope value, n (1.0), was changed.

Protection by Allosteric Effectors against Inactivation. A number of activators, inhibitors, and analogues were surveyed for their ability to protect the enzyme against inactivation. All of the effectors tested afforded some protection, as indicated by the increase in the time required for half-inactivation (Table III). Fructose-P_2 was quite efficient at low concentration.

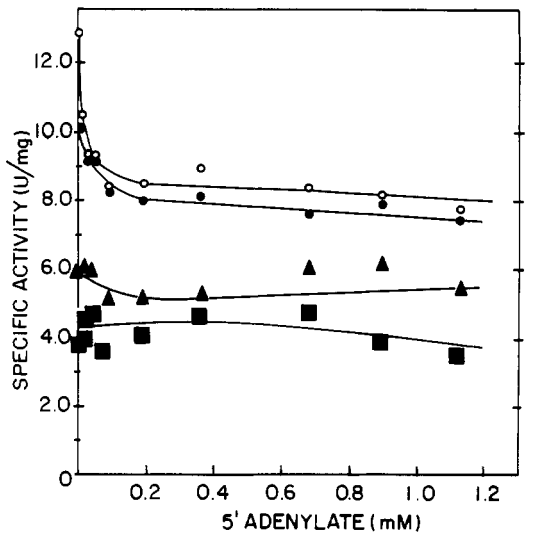

FIGURE 6: Inhibition of [^{14}C]PG-modified enzyme by 5′-AMP. The inhibition of synthesis activity was examined by using the enzyme prepared as in Table II with the following [^{14}C]PG content per subunit (mol of [^{14}C]PG/mol of subunit): none (○); 0.12 (●); 0.43 (▲); 1.1 (■). The assay was done in the absence of fructose-P_2 but with increased concentrations of substrates (i.e., 0.2 μmol of glucose-1-P, 1.5 μmol of ATP, and 5.0 μmol of $MgCl_2$) for maximum activity in the absence of activator.

Table III: Protection of Enzyme Activity by Allosteric Activators and Inhibitors and Their Analogues during Reaction with PG[a]

allosteric effector	concn (mM)	$t_{0.5}$ (min)	allosteric effector	concn (mM)	$t_{0.5}$ (min)
none		20	5′-adenylate	0.5	27
				5.0	47
fructose-P_2	0.5	60	Na_2HPO_4	5.0	20
	5.0	>100		30.0	45
fructose-1-P	2.0	27	$Na_2B_4O_7$	5.0	20
	20.0	>100		30.0	>100
fructose-6-P	1.0	21	Na_2SO_4	5.0	31
	10.0	46		30.0	54
hexanediol-P_2	0.1	90	$Na_4P_2O_7$	5.0	>100
	1.0	>100			

[a] Enzyme (14 μM) in the presence of 2 mM ATP plus 20 mM $MgCl_2$ was incubated with 0.5 mM PG in the presence of various ligands. Aliquots were removed during reaction and assayed for fructose-P_2-stimulated activity in synthesis. The half-life of inactivation ($t_{0.5}$) was determined from semilogarithmic plots of data expressed as the percentage of the initial activity vs. time.

Two analogues of it, fructose-1-P and fructose-6-P, were much less efficient, in keeping with their inability to serve as activators of the *Escherichia coli* enzyme. On the other hand, hexanediol-P_2 was very effective as a protective ligand and has been shown to be efficient as an activator (Haugen et al., 1974; Haugen & Preiss, 1979).

The allosteric inhibitor 5′-AMP also exerted some protective effect, more so than orthophosphate, which is a very weak inhibitor of ADP-glucose synthetase (Preiss et al., 1966). Sodium borate and sodium sulfate gave protection roughly comparable to that of orthophosphate, and pyrophosphate was quite efficient. The incorporation of [^{14}C]phenylglyoxal was not followed in these studies.

Discussion

Fructose-P_2 is the natural allosteric activator of *E. coli* ADP-glucose synthetase and effectively regulates the enzyme activity in vivo (Preiss et al., 1966; Govons et al., 1973). A number of other compounds structurally related to fructose-P_2 also activate the enzyme to varying degrees. They apparently share the same binding site and have in common two phosphoryl groups or one phosphate plus one carboxyl or aldehyde group (Preiss, 1972). This suggests that the activator-binding region of the enzyme contains at least two residues with positively charged side chains in spatial, if not linear, proximity. Chemical modification studies with reagents specific for lysine residues, pyridoxal-P and trinitrobenzenesulfonate, have recently borne out this prediction (Haugen et al., 1976b; Parsons & Preiss, 1978a,b; Carlson & Preiss, 1981). Lys-38 of the amino terminus is absolutely essential for binding of activator, while at least one of several other nearby lysines also plays a critical role in activation by providing the second necessary cationic side chain. The present results with CHD and PG indicate that arginine also provides a cationic side chain critical to allosteric activation.

Arginine residues were implicated by two lines of evidence from the CHD study. The conditions employed favored arginine reaction, and the modification was reversible by hydroxylamine treatment, eliminating any unpredicted irreversible side effects as cause for the inactivation (Patthy & Smith, 1975a). Also, amino acid analysis showed that loss of arginines accompanied modification to a comparable extent. CHD and PG are both highly specific for arginine in terms of the amino acid residue modified (Takahashi, 1968).

The original experiments, that established that the modified arginine derivative consisted of two molecules of PG per residue (Takahashi, 1968), were carried out by using free arginine as a model compound. However, they have been borne out by subsequent protein studies (Lange et al., 1974; Bond et al., 1980; Shoun et al., 1980). In some instances, a stable derivative may be formed by incorporation of only one PG (Borders & Riordan, 1975). This situation may predominate if PG modification is done in the presence of borate, resulting in a PG–borate–arginine complex (Werber et al., 1975; Kazarinoff & Snell, 1976; Marcus, 1976). In the present study, the more generally accepted stoichiometry of 2:1 would suggest the modification of one arginine per subunit by PG. The inactivation reaction order derived kinetically led to the conclusion that reaction of one molecule of PG was sufficient. The results agree, however, if inactivation results from reaction of the first PG molecule with the guanidinium group, forming the initial glyoxaline ring structure postulated (Takahashi, 1968). A second molecule of PG would subsequently form the final derivative but not contribute to the inactivation.

Both the protection study and kinetic studies demonstrated that the region of the enzyme being modified was involved in allosteric regulation. Allosteric activators and inhibitors protected the enzyme from inactivation by either CHD or PG, and with the latter, the protection was comparable to their relative strength as allosteric effectors. PG-modified enzyme activity was decreased in V_{max} and was less responsive to either activation by fructose-P_2 or inhibition by 5′-AMP. Nevertheless, the activity assayed in the absence of fructose-P_2 was essentially unaffected, and therefore modification was not simply inactivating catalysis. The PG studies showed that both the synthesis and pyrophosphorolysis functions of the enzyme were affected and that the decreased affinities for ATP or ADP-glucose were the only substrate functions altered. These are the first of the two ordered substrates to bind in each of the catalytic directions, and the lowered affinities for them would also reflect the loss of stimulation by the fructose-P_2 (Haugen & Preiss, 1979; Preiss et al., 1966).

It is not clear whether the critical arginine residue provides the second cationic side chain postulated as necessary for

binding allosteric activator or if it contributes in a more general way to form an anion binding site. On the one hand, the planar structure and hydrogen-bonding capability of the guanidinum group make it particularly well suited to bind phosphate groups (Cotton et al., 1973). Furthermore, there are a number of examples of arginine directly involved in the binding of fructose-P_2 or pyridoxal-P (Marcus, 1975; Kazarinoff & Snell, 1976; Riordan et al., 1977). This would argue for a direct role in activator binding by ADP-glucose synthetase.

On the other hand, the particular reactivity of lysine-41 in ribonuclease has been ascribed to the proximity of other cationic side chains which together effectively reduce the pK of that ϵ-amino group (Hirs, 1962). Recent studies indicate that arginine-39 is probably one of these (Takahashi, 1968; Patthy & Smith, 1975b). An analogous picture is emerging for rabbit muscle aldolase, where several cationic residues, including lysine-146, arginine-148, and histidine-359, are implicated in the binding of the two phosphate groups of the substrate [Lobb et al. (1976) and references cited therein]. A comparable situation may exist in ADP-glucose synthetase, with lysine-38 being located nearby other essential lysines and arginines in the linear sequence. Together they form an anion binding pocket in the backbone of the enzyme.

The effect of formation of the arginine derivative may be due as much to steric interference with activator binding as it is to neutralization of the positive charge. Patthy & Smith (1975a) pointed out that the derivative formed by CHD reaction is still protonated unless borate is also present and that this distinction could be useful in discriminating between electrostatic and steric effects. The initial inactivation by CHD occurred in the absence of borate and was not enhanced by it, although the derivative was stabilized. This would suggest that the accessibility of the arginine was important, as well as its protonation. In lysozyme, inactivation by CHD also does not absolutely depend upon borate, although it is enhanced 2-fold by its presence (Patthy & Smith, 1975b). We found that PG was much more effective as an inactivator than CHD, besides being highly specific. The PG–arginine derivative is a very large, hydrophobic heterocyclic compound (Takahashi, 1968). Therefore, its greater effectiveness with ADP-glucose synthetase was probably due to both charge neutralization and steric hindrance.

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