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Modification of the Allosteric Activator Site of *Escherichia coli* ADP-glucose Synthetase by Trinitrobenzenesulfonate†

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ABSTRACT: Limited modification of *Escherichia coli* B ADP-glucose synthetase (EC 2.7.7.27) by trinitrobenzenesulfonate (TNBS) appeared to affect primarily the allosteric properties of the enzyme. There was little loss of the catalytic activity assayed in the absence of activator. However, the abilities of fructose 1,6-bisphosphate or hexanediol 1,6-bisphosphate to activate the enzyme, or of 5′-adenylate to inhibit the enzyme, were rapidly lost upon trinitrophenylation. Modification progressively decreased the affinity for activator, decreased the V_{max} at saturating concentrations of activator, and decreased the cooperativity among activator binding sites. These effects could be completely prevented by the presence of allosteric effectors during reaction with TNBS, although a low amount of trinitrophenylation still occurred. Substrates partially protected the enzyme from reaction with TNBS. The lysyl ε-amino side chain was modified by trinitrophenylation, but the target was not primarily the same residue which could form a Schiff base with pyridoxal phosphate, another activator of the enzyme. A large peptide containing most of the trinitrophenyl residue was isolated after cleavage of the enzyme and was identified as part of the N-terminal amino acid sequence. The migration of the enzyme on polyacrylamide gel electrophoresis or on agarose column chromatography was unchanged by modification. However, the ability of fructose-1,6-P_2 to induce the oligomerization of a mutant form of the enzyme was completely prevented by trinitrophenylation. This effect could be protected against by the presence of activator or inhibitor during reaction with TNBS.

ADP-glucose synthetase (EC 2.7.7.27) catalyzes the rate-limiting step in bacterial glycogen biosynthesis, the formation of a glucosyl donor (Preiss, 1969, 1973, 1978). 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-P_2 (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-P 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 were also initiated with 2,4,6-trinitrobenzenesulfonate (TNBS),[1] another reagent specific for amino groups. This report demonstrates the presence of additional lysine residue(s) essential to allosteric regulation which preferentially react with TNBS. The major TNP-lysine-containing peptide was isolated after cleavage of the modified enzyme with cyanogen bromide, and its identification is described.

Experimental Procedures

Reagents. TNBS, purchased from Sigma, was recrystallized from dilute HCl. α-Dinitrophenylvaline was from Sigma. α-TNP-valine and ε-TNP-lysine (Okuyama & Satake, 1960; Kotaki & Satake, 1964), *S*-TNP-cysteine (Hollenberg et al., 1971), [^{3}H]pyridoxal-5′-P (Haugen & Preiss, 1979; Stock et al., 1966), and hexanediol-P_2 (Hartman & Barker, 1965) were synthesized as described. Spectrapor 3 dialysis tubing was a product of Spectrum Medical Industries. Fluorescamine was purchased as Fluram from Roche Diagnostics. Trypsin treated with 1-(tosylamido)-2-phenylethyl chloromethyl ketone was from Worthington. All other reagents were of the highest possible commercial grade.

ADP-glucose Synthetase Assays (Synthesis Direction). (*a*) *Activated Conditions.* Assay A-1 for measuring 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]-

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[1] Abbreviations used: TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, 2,4,6-trinitrophenyl; [^{3}H]pyridoxal-P, [4′-^{3}H]pyridoxal 5′-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate; FDNB, 1-fluoro-2,4-dinitrobenzene.

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glucose-1-P [(5–10) × 10^5 cpm/μmol], 0.3 μmol of ATP, 1.0 μmol of $MgCl_2$, 0.5 μg of yeast inorganic pyrophosphatase, allosteric activator as indicated, and enzyme. Saturating concentrations of activator enhance the enzyme activity 35-fold.

(*b*) *Unactivated Conditions* (*Assay A-2*). In the absence of activator, the optimum amounts of substrates were 0.2 μmol of glucose-1-P, 1.5 μmol of ATP, and 5.0 μmol of $MgCl_2$.

ADP-glucose Synthase Assay (*Pyrophosphorolysis Direction*). (*a*) *Activated Conditions*. Assay B-1 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) × 10^6 cpm/μmol], and the activator or inhibitor indicated.

(*b*) *Unactivated Conditions* (*Assay B-2*). In the absence of activator, the amounts of substrates were increased to 0.7 μmol of ADP-glucose and 5.0 μmol of $MgCl_2$.

All enzyme assays were done at 37 °C and initiated by the addition of enzyme. All assays were done with the 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 $I_{0.5}$, the concentration of substrate, activator, or inhibitor required for half-maximal velocity, activation, or inhibition, respectively; $\bar{n}$, the Hill interaction coefficient (Taketa & Pogell, 1965; Changeux, 1963; Atkinson et al., 1965).

Isolation of Enzyme. The homogeneous ADP-glucose synthetase used in this study was predominantly from strain AC70R1 of *E. coli* B, prepared as described (Haugen et al., 1976a). Several experiments utilized the enzyme from strain SG5-504, which was purified by a modification (Carlson et al., 1976) of the basic procedure.

Reaction with Trinitrobenzenesulfonic Acid. Enzyme was freed of 1,4-dithioerythritol in the storage buffer by dialysis against 0.05 M Hepes buffer, pH 7.0, containing 0.24 mM EDTA. It was treated with a fresh solution of TNBS in 0.05 M Mops buffer, pH 8.0, at 25 °C unless otherwise noted, under reduced room light. Aliquots for assay of enzyme activity were removed at time points and diluted 1000-fold into 0.05 M Mops buffer, pH 8.0, containing 1 mM L-lysine. Lysine had no effect on the enzyme kinetics.

During the reaction, the progress of trinitrophenylation was measured directly in the Beckman DUR spectrophotometer by the absorbance at 367 nm, assuming a molar extinction coefficient of 10.8 × 10^3 (Goldfarb, 1966; Coffee et al., 1971). Trinitrophenyl group incorporation was also measured by the absorbance at 345 nm, assuming a molar extinction coefficient of 14.0 × 10^3 (Goldfarb, 1966), after the enzyme was recovered by addition to the reaction mixture of 3 volumes of cold saturated $(NH_4)_2SO_4$ and extensive dialysis in the dark against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.25 M EDTA.

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 with the relation A^{1cm}_{280nm} = 1.0 mg/mL (Haugen et al., 1976b), but interference from the trinitrophenyl group absorbance became significant with extensively modified enzyme.

Incorporation of [^{3}*H*]*Pyridoxal-P.* The procedure for the reaction of enzyme with [^{3}H]pyridoxal-P, and subsequent reduction of the Schiff's base with $NaBH_4$, followed that described by Haugen et al. (1976b) for reaction with unlabeled pyridoxal-P. However, after reduction, samples were dialyzed extensively against 0.1 M K_2HPO_4 buffer, pH 7.0, containing 0.25 mM EDTA. Protein was determined by the absorbance at 280 nm, and an aliquot was removed for counting in PCS, the liquid scintillation solution of Amersham/Searle.

Identification of ε-TNP-lysine. Enzyme that had been trinitrophenylated was dialyzed against 10 mM KH_2PO_4, pH 7.0. Hydrolysis was done in sealed, evacuated vials containing 5.7 N HCl at 110 °C for 16 h. The HCl was removed by evaporation under a stream of nitrogen at 40 °C and the residue dissolved in a small volume of ethyl acetate–1-butanol–acetic acid (100:100:1) (Hollenberg et al., 1971). The extract was spotted on Whatman no. 1 paper. The paper was saturated with 0.10 M sodium citrate, pH 5.0, and subjected to electrophoresis for 90 min at 600 V (55 mA) in 1.6 M formic acid. The paper was dried, and areas containing trinitrophenylated amino acids, evident under both visible and ultraviolet light, were cut out. The trinitrophenyl amino acids were eluted into 1% $NaHCO_3$ and quantitated by the absorbance at 345 nm. Recovery was corrected by use of standard α-TNP-valine, ε-TNP-lysine, and *S*-TNP-cysteine. Alternatively, the paper was stained with ninhydrin following electrophoresis. Trinitrophenyl amino acids were also quantitated after descending chromatography on Whatman no. 1 paper in 1.5 M NaH_2PO_4, pH 5.4.

Determination of Sulfhydryl Content and N-Terminal Residue. Free sulfhydryl content was measured by the reaction of enzyme with DTNB in the presence of $NaDodSO_4$ (Ellman, 1959). The N-terminal amino acid reactivity was assayed with FDNB by the basic method of Sanger (Sanger & Thompson, 1953; Narita et al., 1975). Dinitrophenyl amino acids were chromatographed along with standard DNP-valine on silica gel 1B plates in benzene–pyridine–acetic acid (80:20:2). They were quantitated by measuring the absorbance at 350 nm in 1% $NaHCO_3$, and recovery was corrected by use of the standard.

Estimation of Enzyme Molecular Size. The behavior of modified enzyme during gel permeation column chromatography in the presence of fructose-P_2 was examined as described (Carlson et al., 1976). Its migration on polyacrylamide gels in the absence of $NaDodSO_4$ was measured with the electrophoresis system of Hedrick & Smith (1968) followed by staining with Coomassie Brilliant Blue (Chrambach et al., 1967).

TNP-peptide Isolation. For the large-scale modification, 30 mg of enzyme in 10 mL of 0.1 M $NaHCO_3$ buffer, pH 8.0, along with 2 mM ADP-glucose and 20 mM $MgCl_2$, was treated with 0.75 mM TNBS for 10 min. After precipitation with $(NH_4)_2SO_4$, enzyme was dialyzed against 0.05 M $NaHCO_3$ buffer, pH 8.0, containing 0.25 mM EDTA.

Trinitrophenylated enzyme, at a concentration of 5 mg/mL, was heat denatured in a boiling water bath for 5 min, and the precipitated enzyme was solubilized in 1.5 mL of 90% formic acid. Solid cyanogen bromide was added in a 100-fold excess over the enzyme molar methionine content, and the reaction tube was tightly covered and left at room temperature for 20 h. Isolation of the resulting peptides was generally approached by the method of Fowler (1975). The peptide mixture was first resolved by column chromatography on Sephadex G50 in 30% acetic acid. The sample in formic acid was diluted to 3.0 mL with 30% acetic acid and applied to a 2.5 × 20 cm

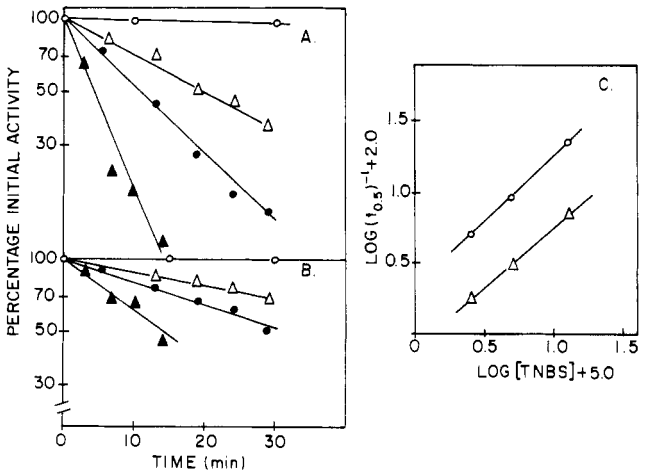


FIGURE 1: Inactivation of fructose-P_2-stimulated enzyme activity by TNBS. The loss with time of synthesis (A) and pyrophosphorolysis (B) activities of 7 μM enzyme subunits incubated at 37 °C with the following concentrations of TNBS: none (○); 25 μM (△); 50 μM (●); 125 μM (▲). (C) Determination of the order of the inactivation reaction: synthesis (○); pyrophosphorolysis (△).

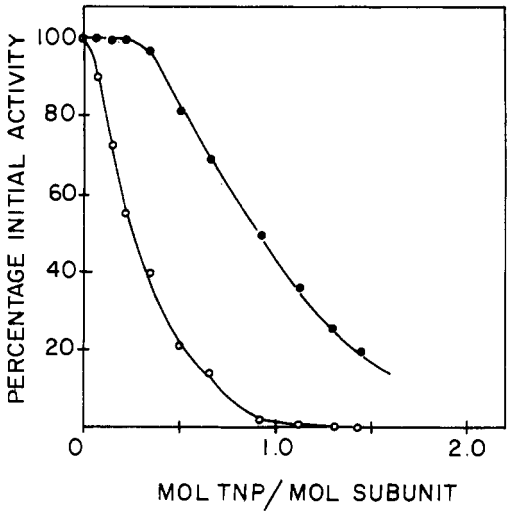


FIGURE 2: Incorporation of trinitrophenyl (TNP) groups accompanying inactivation. The titration of synthesis (○) and pyrophosphorolysis (●) activities of 15 μM enzyme subunits incubated with 160 μM TNBS for up to 60 min. After various incubation times, samples of the reaction mixture were removed and assayed for enzyme activity and for the amount of trinitrophenyl-bound enzyme formed. The derivative was assayed by the absorbance at 367 nm.

column of Sephadex G50 equilbrated with 30% acetic acid, and fractions of 2.3 mL were collected at 60 mL/h. The pooled fractions were concentrated by flash evaporation and then dialyzed against two 100-mL portions of 8 M urea. The pH was adjusted to 5.0 with dilute NH_4OH, and then dialysis was continued against 150 mL of 0.02 M sodium acetate buffer, pH 5.0, containing 8 M urea. This and all subsequent dialyses employed Spectrapor 3 membrane tubing. The peptide sample was now applied to a 1.0 × 17 cm column of CM-cellulose equilibrated with the same buffer. After the column was washed with 15 mL of buffer, a 140-mL linear gradient of 0–0.5 M NaCl in buffer was initiated, and 2.8-mL fractions were collected at 20 mL/h. The pooled fractions were dialyzed against 0.04 M ammonium formate buffer, pH 3.4, containing 8 M urea, and chromatographed on a column of SP-Sephadex, equilibrated with the same buffer. Fractions were collected after initiation of a linear gradient of 0–0.5 M NaCl in buffer. The pooled fractions were dialyzed twice against 1 L of 10% acetic acid.

For estimation of both homogeneity and molecular size of the large peptides, polyacrylamide gel electrophoresis was carried out by using gels containing urea and $NaDodSO_4$ with insulin, myoglobin, and cytochrome *c* as standards (Swank & Munkries, 1971). Amino acid analysis was carried out on a Durrum D-500 analyzer. TNP-lysine could not be detected on this resin by employing the standard program. The amino-terminal amino acid squence was determined by automated Edman degradation using a Beckman 890C sequencer. N-Acetylated cytochrome *c* was added to the sample as carrier, and the standard program employing dimethylallylamine buffer was employed. The cleaved residues were identified by gas chromatography and thin-layer chromatography as described (Carlson et al., 1976).

For digestion with trypsin, the cyanogen bromide derived peptides were extensively dialyzed against 0.05 M $NaHCO_3$ buffer, pH 8.5. To the opalescent solution was added trypsin at a 1:50 ratio (w/w) in three aliquots at 2-h intervals, and incubation at 37 °C was continued for 20 h. The sample was applied directly to a column of DEAE-cellulose equilibrated with 0.025 M $NaHCO_3$, pH 8.5, and eluted with a linear gradient of 0.025–1.0 M $NaHCO_3$. Peptides were detected by reaction with fluorescamine (Udenfriend et al., 1972). Pooled fractions were desalted on columns of Sephadex G25 in 10% acetic acid.

Results

Inactivation Kinetics. TNBS rapidly inactivated ADP-glucose synthetase at pH 8.0 in Mops buffer (Figure 1). The loss of activity followed pseudo-first-order kinetics, giving a linear plot of activity vs. time on a semilogarithmic graph. The rate of inactivation measured in the synthesis direction (see Experimental Procedures) was 3 times faster than that in the direction of pyrophosphorolysis. The half-life for either reaction was proportional to the concentration of TNBS ranging up to a 75-fold molar excess over the concentration of enzyme. The apparent first-order rate constants under the conditions shown varied from 0.035 to 0.154 min^{-1} in synthesis and from 0.012 to 0.046 min^{-1} in pyrophosphorolysis. The corresponding second-order rate constants for synthesis and pyrophosphorolysis were 5.7×10^8 and 1.8×10^8 min^{-1} M^{-1}. The reaction order for the inactivation process with respect to TNBS concentration was determined by plotting log (half-time inactivation)$^{-1}$ vs. log (TNBS) after the method of Lévy et al. (1963) and others (Hollenberg et al., 1971; Scrutton & Utter, 1965; Keech & Farrant, 1968). The slope (n) indicated the number of molecules of TNBS reacting with each active site (subunit) of the enzyme (Figure 1C). The values $n = 0.90$ and $n = 0.95$ for synthesis and pyrophosphorolysis suggested that the loss of either activity resulted from reaction of only one molecule of TNBS per enzyme subunit.

When the modification by a given concentration of TNBS was followed spectrophotometrically, there was indeed complete loss of synthesis activity accompanying the incorporation of one trinitrophenyl group per M_r 50 000 enzyme subunit (Figure 2). However, pyrophosphorolysis activity again appeared less sensitive to the effects of modification. About 0.3 trinitrophenyl group per subunit could be incorporated before any activity was lost, and the complete loss of pyrophosphorolysis activity required nearly two trinitrophenyl groups incorporated per subunit.

Modification continued beyond the point of complete inactivation, approaching a maximum of three trinitrophenyl groups per subunit under the given conditions.

Effect of Modification on Activity. Fructose-P_2-stimulated catalytic activity was most affected by TNBS. When synthesis or pyrophosphorolysis was assayed with the optimum con-

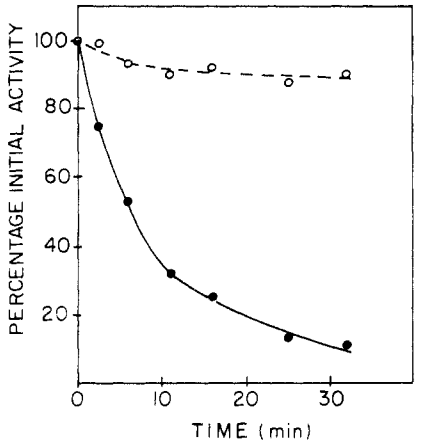


FIGURE 3: Effect of TNBS upon fructose-P_2-stimulated activity. Enzyme (7.5 μM) was incubated with 100 μM TNBS for the times indicated before activity was assayed in synthesis under fructose-P_2-activated (●) and unactivated (○) conditions. Initial specific activities were 48.1 and 19.9 units/mg, respectively.

Table I: Effect of Modification on Response to Activators[a]

mol of TNP/ mol of subunit	fructose-P_2			hexanediol-P_2		
	V_{max} (%)	$A_{0.5}$ (mM)	$\bar{n}$	V_{max} (%)	$A_{0.5}$ (μM)	$\bar{n}$
0	100	0.08	1.8	100	0.0032	2.1
0.35	45	0.12	1.5	51	0.0049	1.8
0.52	22	0.20	1.15	19	0.0059	1.6

[a] Enzyme (7 μM) was incubated with 125 μM TNBS for varying times. The activator saturation was then examined in two experiments. Assay was in the direction of synthesis, and kinetic parameters were derived from Hill plots. The specific activities represented by V_{max} were 53.8 and 58.0 units/mg for the two experiments.

centration of substrates in the absence of the allosteric activator (assay A-2 or B-2), there was negligible inactivation (Figure 3). By contrast, activity as normally assayed in the presence of fructose-P_2 was rapidly lost. Thus, a primary effect of trinitrophenylation was to interfere with the normal allosteric activation of the enzyme.

This effect was further characterized for fructose-P_2 and its analogue 1,6-hexanediol-P_2, which is an extremely effective activator of ADP-glucose synthetase (Haugen et al., 1974; Haugen & Preiss, 1979). The saturation by fructose-P_2 or hexanediol-P_2 was examined for enzyme modified to varying degrees. As the extent of modification increased, the maximum velocity for synthesis attainable with saturating concentrations of either activator was reduced (Table I). Furthermore, the concentration of activator required for half-maximal activation increased about 2-fold. At the same time, the Hill coefficient $\bar{n}$, denoting the strength of interaction between subunits, decreased significantly (Table I). The trend, therefore, was that as modification approached one trinitrophenyl group per subunit, the cooperativity between subunits for activation was lost and the apparent affinity for activator decreased severalfold. The net result was the loss of activator-stimulated activity as shown above (Figure 3).

These results were supplemented by an examination of substrate saturation kinetics of unmodified and modified partially active enzyme preparations (data no shown). In the direction of synthesis, the V_{max} was decreased 2–3-fold. The $S_{0.5}$ value for glucose-1-P was unchanged, but for ATP, it was increased about 50%, to 0.66 mM. The Hill coefficient ($\bar{n}$) for either substrate was not significantly changed. In pyrophosphorolysis, the V_{max} was again decreased. The $S_{0.5}$, for

Table II: Extent of Modification of the Enzyme by TNBS in the Presence of Activator or Substrate[a]

ligand present	$t_{0.5}$ (min)	mol of TNP/ mol of subunit at 20 min
none	1	2.2
fructose-P_2	11	1.7
ADP-glucose	12	1.2
fructose-P_2 + ADP-glucose	68	1.0

[a] Enzyme (10 μM) was incubated for up to 20 min with 0.90 mM TNBS alone or along with 0.90 mM fructose-P_2 or 1.6 mM ADP-glucose. The half-life ($t_{0.5}$) for loss of synthesis activity was derived from semilog plots of each time course.

Table III: Protection of Enzyme Activity by Ligands during Reaction with TNBS

allosteric effector[a]	concn (mM)	$t_{0.5}$[b] (min)	substrate[a]	concn (mM)	$t_{0.5}$[b] (min)
none		7	none		6
fructose-P_2	0.5	16	ATP	1.0	11
				5.0	32
	5.0	110			
			ATP + $MgCl_2$	1.0, 5.0	6
NADPH	0.5	28			
			glucose-1-P	0.5	7
	5.0	>150		5.0	10
AMP	0.5	100	PP_i	0.5	8
				5.0	11
	5.0	150			
			ADP-glucose	1.0	8
P_i	5.0	8		5.0	22
	50.0	33	ADP-glucose + $MgCl_2$	1.0, 5.0	8
			$MgCl_2$	5.0	6

[a] The respective ligand was present during reaction of 2 μM enzyme subunits with 0.20 mM TNBS at 37 °C. [b] 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.

ADP-glucose was increased 4-fold, from 0.09 to 0.33 mM, but the $S_{0.5}$ for PP_i was unchanged by trinitrophenylation. The Hill coefficients for both of these substrates were unchanged.

Stoichiometry of Trinitrophenylation. Although the inactivation in synthesis could be accounted for by the incorporation of only one trinitrophenyl group per subunit (Figure 2), up to two more trinitrophenyl groups could be incorporated. The distribution of this additional modification in the presence of fructose-P_2 and ADP-glucose and its effect upon the catalytic activity were examined. The protective effect of these two ligands was accompanied by a significant decrease in trinitrophenylation (Table II). Their combined effect is consistent with the finding from equilibrium dialysis studies that activator and substrate bind to the enzyme synergistically (Haugen & Preiss, 1979). It was still possible to incorporate about one trinitrophenyl group even when the catalytic activity was greatly protected (Table II).

Protection against Inactivation. A number of other allosteric effectors and substrates of the enzyme were also examined as protecting agents during reaction with TNBS. All of the ligands except $MgCl_2$ could protect to some extent against inactivation, as indicated by an increase in the time required for loss of half the initial activity (Table III). The extent of protection was proportional to the concentration of ligand. There was very efficient protection by the allosteric activators fructose-P_2 and NADPH, and by the allosteric inhibitor 5′-AMP. The high concentration of P_i required for any protective

Table IV: Effect of Modification in the Presence of Substrate upon Response to Allosteric Effectors[a]

mol of TNP/ mol of subunit	fructose-P_2			AMP		
	V_{max} (%)	$A_{0.5}$ (mM)	$\bar{n}$	$V_{initial}$ (%)	$I_{0.5}$ (mM)	$\bar{n}$
0	100	0.11	1.6	100	0.10	1.8
0.4	51	0.12	1.7	45	0.10	1.8
0.6	28	0.13	1.6	37	0.11	1.4
1.0	16	0.12	1.7	25	0.11	1.0

[a] After preparation of modified enzyme by incubation of 20 μM enzyme with 0.74 mM TNBS in the presence of 1.9 mM ADP-glucose and 20 mM $MgCl_2$, the activation by fructose-P_2 was assayed in the synthesis direction. At maximum activation, the specific activity was 69.6 units/mg. In a separate similar experiment, inhibition by AMP in the presence of 1.5 mM fructose-P_2 was examined. The initial specific activity was 62.0 units/mg.

effect was consistent with P_i being a relatively poor inhibitor of ADP-glucose synthetase (Preiss et al., 1966). Even though $MgCl_2$ had little demonstrable effect here, it was included in further substrate protection experiments because it has been shown to be involved in catalysis both alone and in conjunction with the other substrates.

Modification in the Presence of Substrate. Since the presence of substrates reduced the reactivity of the enzyme toward TNBS, the allosteric properties of the enzyme were reexamined after modification in the presence of substrates. Under these conditions, a reactive residue necessary for catalysis would presumably no longer be a possible target for trinitrophenylation. High concentrations of ADP-glucose and $MgCl_2$ were used, as required for completely unactivated enzyme (assay A-2). The rationale for this was that a progressive interference by TNBS with allosteric activation could have a secondary influence upon the effectiveness of protection by substrates at lower concentrations than these.

The reaction with TNBS now proceeded more slowly, as expected, but there was still incorporation of between one and two trinitrophenyl groups per subunit accompanying loss of all activated enzyme activity. Enzyme that had been progressively modified was examined for fructose-P_2 activation and AMP inhibition. The effect upon the fructose-P_2-stimulated activity of partially active enzyme preparations was limited to a reduction in the V_{max} (Table IV). Hill plots showed that the response of the residual activities to fructose-P_2 was normal with respect to the $A_{0.5}$ and subunit cooperativity. The difference between these results and those of Table I, where modification was done in the absence of substrate, may reflect interaction between the catalytic and regulatory sites of the enzyme, to be discussed below. There was a complex effect upon inhibition by AMP. As assayed in the presence of fructose-P_2 (Table IV), the inhibition of the modified preparations followed the same kinetics as those of the unmodified enzyme: both the $I_{0.5}$ and $\bar{n}$ values, at less than one trinitrophenyl group per subunit, were the same as those for the unmodified enzyme. However, when activity was assayed in the absence of fructose-P_2 (Figure 4), the ability of AMP to inhibit the enzyme was progressively lost. At one trinitrophenyl group per subunit, there was no inhibition by even very high concentrations of AMP, and there was in fact some apparent activation.

Identification of the Modified Residues. The acid hydrolysate of the modified enzyme was subjected to paper electrophoresis and paper chromatography in comparison to standard ε-TNP-lysine and *S*-TNP-cysteine. The trinitrophenyl derivative in the hydrolysate was thus identified as ε-TNP-lysine. ε-TNP-lysine alone was quite stable to acid

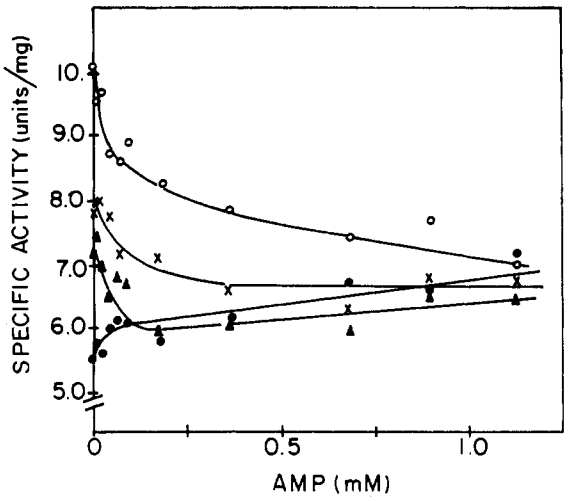


FIGURE 4: AMP inhibition of modified enzyme. The inhibition by AMP in the absence of fructose-P_2 (assay A-2) was examined on the enzyme prepared as described in Table IV with the following TNP content per subunit (moles of TNP per mole of subunit): none (○); 0.4 (×); 0.6 (▲); 1.0 (●).

hydrolysis, with an average of 85 ± 8% recovered in three experiments. It was partially degraded when hydrolyzed in the presence of protein, however (Coffee et al., 1971; Freedman & Radda, 1969). When ε-TNP-lysine and bovine serum albumin or ADP-glucose synthetase were hydrolyzed together, only 70 ± 4% and 77 ± 5%, respectively, of the ε-TNP-lysine was recovered. When modified ADP-glucose synthetase (1.2–2.6 trinitrophenyl groups per subunit) was hydrolyzed, the amount of ε-TNP-lysine recovered after chromatography of the extract accounted for 60 ± 9% of the original amount determined before hydrolysis.

The possibility of cysteine being trinitrophenylated (Kotaki et al., 1964) was also considered by examining the reactivity of sulfhydryl groups to dithionitrobenzoic acid after the enzyme was modified. Five preparations of enzyme with 1.0–2.6 trinitrophenyl groups per subunit were found to contain 6.9 ± 0.4 sulfhydryl groups per subunit, compared to an average of 7.1 ± 0.4 sulfhydryls from three determinations upon unmodified enzyme. Previously published values with DTNB have indicated 7.6 ± 0.2 sulfhydryls per subunit of ADP-glucose synthetase (Haugen et al., 1976a; Carlson et al., 1976).

In order to examine the possible modification of the N-terminal α-amino group, which in ADP-glucose synthetase is a valyl residue (Haugen et al., 1976a; Carlson et al., 1976), it was necessary to measure it indirectly. The dinitrophenyl derivative of α-amino groups is quite stable to acid hydrolysis while the trinitrophenyl derivative is not (Kotaki & Satake, 1964; Narita et al., 1975). The availability of the N-terminal valine for reaction with fluorodinitrobenzoic acid was examined before and after modification of the enzyme with TNBS. The hydrolysate of enzyme modified to 2.1 trinitrophenyl groups per subunit still contained α-DNP-valine, although only at about 45% that of the control enzyme.

Reaction of Modified Enzyme with Pyridoxal-P. Pyridoxal-P is an allosteric activator of ADP-glucose synthetase (Gentner et al., 1969). It has been shown that pyridoxal-P can be covalently linked to the enzyme by $NaBH_4$ reduction of a Schiff's base formed with a lysine resiude near the activator binding site (Haugen et al., 1976b; Parsons & Preiss, 1978a). There is a second residue reactive with pyridoxal-P which is protected by substrates and may be at the catalytic site of the enzyme (Parsons & Preiss, 1978a,b). It was of interest to see whether there was any interactive effect between modification with pyridoxal-P and with TNBS.

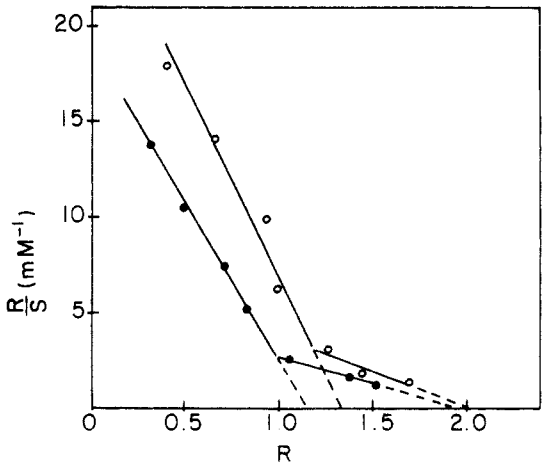


FIGURE 5: Pyridoxal-P binding to modified enzyme. Enzyme was first modified with TNBS in the presence of 1.6 mM ADP-glucose and 1.5 mM $MgCl_2$. Enzyme (4 μM), either unmodified (O) or with 1 mol of TNP/mol of subunit (●), was then incubated at 37 °C in the dark with increasing concentrations of [^{3}H]pyridoxal-P in the presence of 0.5 mM ADP-glucose and 8.0 mM $MgCl_2$ for 30 min before reduction with $NaBH_4$. The data shown in the Scatchard plot are the average of duplicate determinations done at each concentration of [^{3}H]pyridoxal-P.

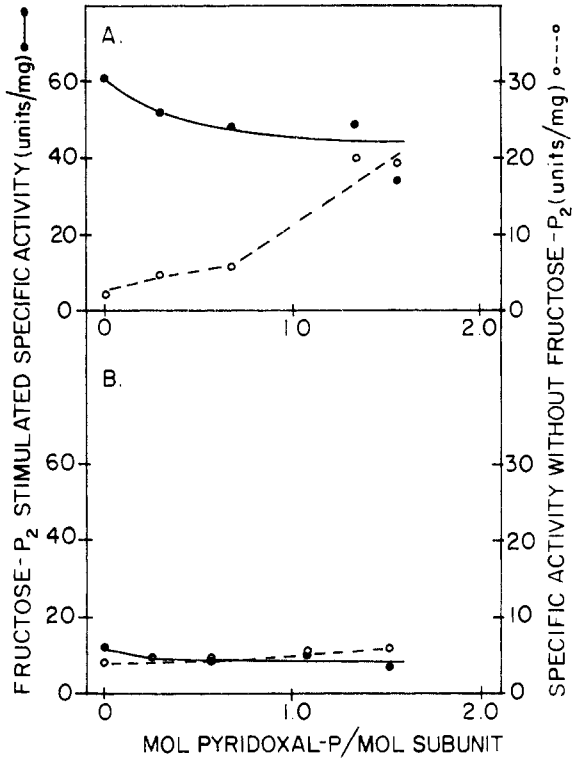


FIGURE 6: Effect of pyridoxal-P incorporation on the activity of modified enzyme. Enzyme prepared as described in Figure 5 was assayed in synthesis (assay A-1) with and without fructose-P_2. (A) Unmodified enzyme. (B) Enzyme with 1 mol of TNP/mol of subunit.

Unmodified enzyme and enzyme containing 1.0 trinitrophenyl group per subunit were exposed to varying concentrations of [^{3}H]pyridoxal-P followed by $NaBH_4$ reduction, and their saturation kinetics were compared. Such an experiment done in the presence of ADP-glucose, to protect the catalytic site, is shown in Figure 5. Although these were not true equilibrium binding conditions, the data were plotted according to Scatchard (1949) and suggested (Figure 5) that under these conditions there was one site per subunit with a high apparent affinity (K_d = 48 μM) for pyridoxal-P and another with a much lower apparent affinity (K_d = 266 μM). The presence

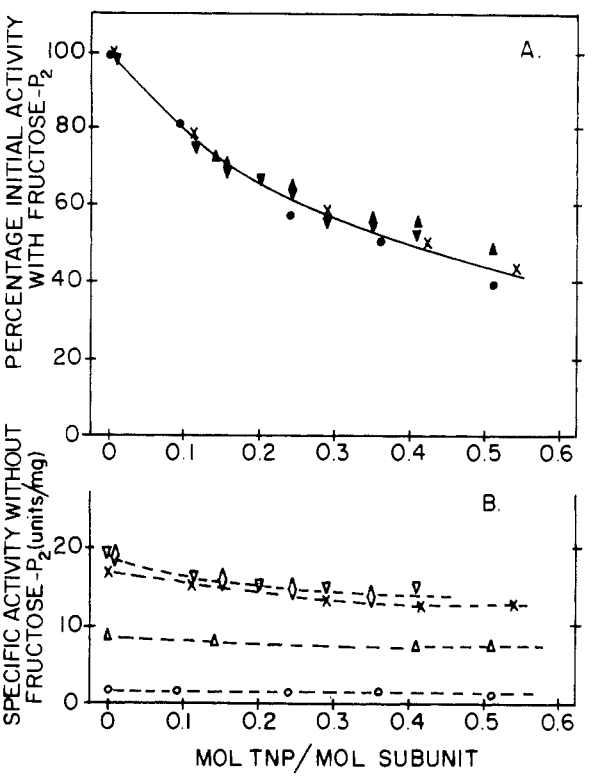


FIGURE 7: Trinitrophenylation of enzyme with bound pyridoxal-P. Both modifications were done in the presence of 0.5 mM ADP-glucose and 8 mM $MgCl_2$. TNBS (0.3 mM) was incubated with 13 μM enzyme containing the following amounts of [^{3}H]pyridoxal-P (mol/mol of subunit): none (●, O), 0.41 (▲, △), 0.71 (×), 1.07 (◆, ◆), 1.38 (▽, ▼). Enzymatic activity, assayed in the synthesis direction in the presence of fructose-P_2, is represented as the percentage of specific initial activity of each preparation: 59.2, 41.2, 34.7, 32.0, and 33.2 units/mg, respectively.

of one trinitrophenyl group per subunit had little effect either on the number of sites available to pyridoxal-P or on their apparent affinities. The results of a number of experiments (not shown) with varying concentrations of substrate supported this conclusion.

In terms of enzyme activity, covalently attached pyridoxal-P is known to effectively stimulate the enzyme in the absence of fructose-P_2 (Haugen et al., 1976b). There was a progressive increase in enzyme activity with attachment of pyridoxal-P (Figure 6A), with little effect upon the fructose-P_2-stimulated activity (assay A-1). The ratio of activities with and without fructose-P_2 decreased from 28 to 1.5, reflecting the activation instead by the bound pyridoxal-P. In contrast, trinitrophenylated enzyme was only slightly stimulated by either activator (Figure 6B). Trinitrophenylation decreased only the activity assayed in the presence of fructose-P_2, consistent with earlier results (Figure 3). However, the attachment of up to 1.5 pyridoxal-P groups per subunit gave very little activation (Figure 6B). Thus, although the attachment of pyridoxal-P to trinitrophenylated enzyme followed nearly normal kinetics (Figure 5), the bound pyridoxal-P could not now stimulate the enzyme activity.

Modification of enzyme by the two reagents in the reverse order was also examined. Enzyme containing varying amounts of bound [^{3}H]pyridoxal-P was exposed to TNBS in the presence of substrate. The rate of trinitrophenylation was not significantly changed by the presence of pyridoxal-P, varying between 0.020 and 0.026 trinitrophenyl group per subunits per min (not shown). Furthermore, trinitrophenylation still progressively reduced the fructose-P_2-stimulated catalytic activity (Figure 7A). The percentage inactivation vs. incorporation

Table V: Effect of Modification on Fructose-P_2-Induced Oligomerization of Mutant SG5-504 Enzyme[a]

expt	protecting ligand	mol of TNP/mol of subunit	activity (%)	elution profile
1	none	1.2	2	A
2	ADP-glucose	0.8	40	A
3	fructose-P_2	0.9	70	B
4	hexanediol-P_2	0.6	100	C
5	ADP-glucose + hexanediol-P_2	0.5	100	D
6	ADP-glucose + AMP	0.1	100	D

[a] Enzyme (15 μM) was exposed to 0.25 mM TNBS for 60 min in the presence of ligands in the following concentrations: 1.5 mM fructose-P_2; 1.8 mM ADP-glucose; 0.25 mM hexanediol-P_2; or 0.9 mM AMP. Enzyme was assayed for TNP content and fructose-P_2-stimulated activity (assay A-1) and applied to gel filtration columns. The elution profiles are shown in Figure 8. Enzyme which was not modified eluted with a profile similar to profile D.

of trinitrophenyl groups followed essentially the same kinetics for all preparations with bound pyridoxal-P. The presence of bound pyridoxal-P increased the catalytic activity up to 14-fold in the absence of fructose-P_2 (Figure 7B). This activation was only slightly reversed by subsequent incorporation of up to 0.6 trinitrophenyl group per subunit. In other words, enzyme preparations only partially modified by pyridoxal-P, which might be further stimulated by fructose-P_2, remained proportionately susceptible to TNBS inactivation. The activation due to bound pyridoxal-P alone was essentially not susceptible.

In summary, these experiments indicated that the covalent binding of either a pyridoxal-P or a trinitrophenyl group was not impeded by the other but that the subsequent modification did not reverse the effect of the first modifier upon the enzyme activity.

Molecular Properties of Modified Enzyme. Modified enzyme (2.1 trinitrophenyl groups per subunit) was examined for altered molecular size. There was no difference in the elution of the protein or of unstimulated enzyme activity from gel filtration columns, nor in the migration through polyacrylamide electrophoresis gels, of modified and unmodified enzyme (not shown). Therefore, the limited trinitrophenylation apparently did not significantly change the structural character of the native enzyme tetramer.

An altered form of ADP-glucose synthetase, purified from a mutant strain of *E. coli* B designated SG5-504, was shown to form higher molecular weight oligomers in the presence of fructose-P_2 (Carlson et al., 1976). It was demonstrated that extensive trinitrophenylation in the absence of any protecting ligand abolished the oligomerization (Carlson et al., 1976). This phenomenon was now studied further to establish the specificity of the effect. The mutant enzyme was trinitrophenylated under varying conditions, and its activity and the ability to oligomerize were examined by gel filtration. As with wild-type enzyme, the presence of substrate or activator during reaction with TNBS both protected against loss of activity and reduced the rate of incorporation of trinitrophenyl groups compared to unprotected enzyme (Table V). SG5-504 enzyme, modified in the presence either of hexanediol-P_2 plus ADP-glucose or of AMP plus ADP-glucose, was fully active and eluted largely as oligomer near the void volume of the column (Figure 8, profile D). By contrast, enzyme modified in the presence of ADP-glucose alone lost half its activity and eluted at V_e = 26 mL, as did unprotected enzyme (Figure 8, profile A). This corresponded to the elution position of the

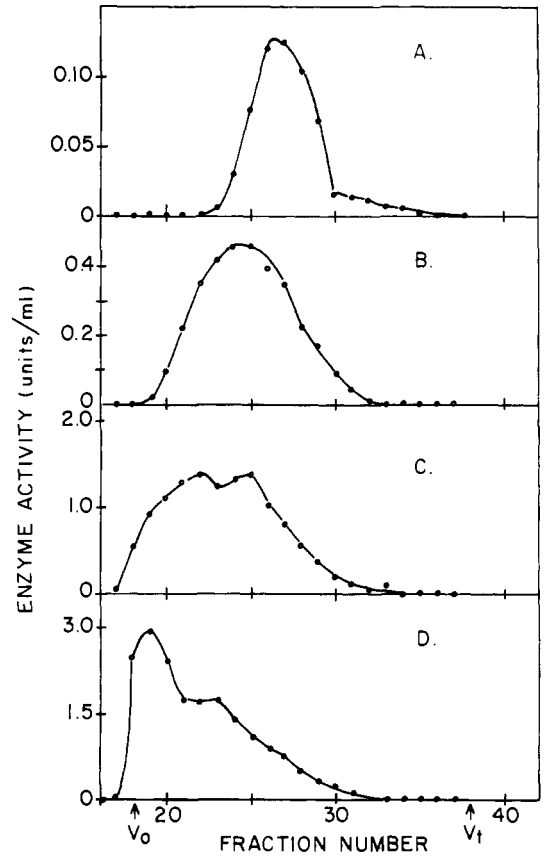


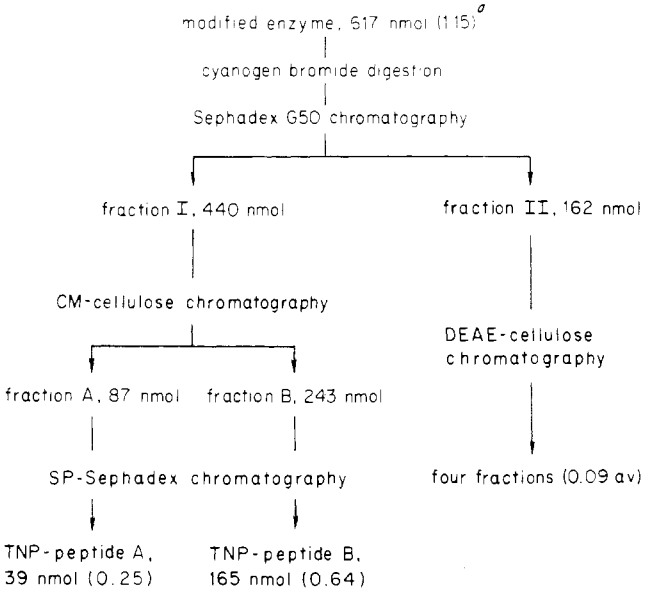
FIGURE 8: Gel filtration of trinitrophenylated SG5-504 enzyme. Enzyme prepared as described in Table V was incubated at 1–2 mg/mL for 10 min at 37 °C in the presence of 1 mM fructose-P_2 before being chilled and applied to a 1.5 × 30 cm Bio-Gel $A_{0.5}$ column (Carlson et al., 1976). The elution buffer (50 mM Tris-HCl, pH 7.2, and 0.5 mM 1,4-dithioerythritol) also contained 1 mM fructose-P_2. The fractions (1 mL) were assayed for synthesis activity (assay A-2, which allows measurement of activity in the complete absence of activator, was used for profiles A and B).

unmodified wild-type form (Carlson et al., 1976).

The effect of modification upon oligomerization was quite specific. For example, with enzyme protected by the presence of ADP-glucose plus hexanediol-P_2, a significant amount of trinitrophenylation still occurred, but the subsequent oligomerization response to fructose-P_2 was completely preserved. Neither ADP-glucose alone, as protecting agent, nor activator alone could preserve the response. Thus, it was the reaction of TNBS with residues which could be protected by the combined presence of substrate and allosteric activator which blocked the oligomerization.

Isolation of TNP-Containing Peptides. The purification of peptides containing trinitrophenylated residues is summarized in Scheme I. The large-scale modification of AC70R1 enzyme was done in the presence of ADP-glucose and $MgCl_2$ to limit the extent of trinitrophenylation. Although enzyme was initially modified to the extent of 1.1 trinitrophenyl groups per subunit, several fractions of cyanogen bromide derived TNP-peptides were in fact separated by Sephadex G50 column chromatography. The major fraction eluted near the void volume of the column. When this fraction I was applied to a CM-cellulose column, it was resolved into two fractions, with the major TNP fraction eluting at about 0.15 M NaCl. When rechromatographed on SP-Sephadex, the major CM-cellulose TNP fraction (fraction B) eluted as a single peak. On Na-DodSO_4–urea electrophoresis gels, this TNP-peptide B was judged to be 90% pure and its molecular size to be about 7400. The amino acid composition of TNP-peptide B (Table VI)

Scheme I: Summary of the Purification of TNP-peptides



[a] The numbers in parentheses indicate nanomoles of TNP per nanomole of peptide.

NH_2 - Leu - Ala - X - Glx - Leu - Lys - Y - Val - Ala - Leu -

FIGURE 9: NH_2-terminal sequence of TNP-peptide B. Residues designated "X" and "Y", which were not identified in this study, were Arg and Ser, respectively, in the amino-terminal sequence of the intact enzyme (Haugen et al., 1976a; Carlson et al., 1976).

showed a great deal of similarity to that of the [^{3}H]pyridoxal-P peptide containing a lysine residue critical to the normal allosteric regulation of the enzyme which was recently isolated and sequenced (Parsons & Preiss, 1978b). The N-terminal amino acid sequence of TNP-peptide B (Figure 9) was determined to be the same as the sequence of the pyridoxylated peptide. Furthermore, the N-terminal leucine residue of TNP-peptide B corresponded to leucine-11 in the sequence of the intact enzyme (Haugen et al., 1976a; Carlson et al., 1976). In an effort to identify the trinitrophenylated lysine residue, the large TNP-peptide B was digested with trypsin. However, the resulting peptide mixture was complex, containing at least four TNP-peptides as resolved by DEAE-cellulose column chromatography.

A minor TNP fraction, fraction A (Scheme I), was recovered from CM-cellulose, eluting at about 0.08 M NaCl. After SP-Sephadex chromatography, this fraction gave a band on $NaDodSO_4$–urea gels corresponding to a molecular size of about 6000 which represented 80–85% of the gel sample. This TNP-peptide A had a much lower TNP content than did TNP-peptide B, and its amino acid composition (Table VI) showed many differences from that of TNP-peptide B, indicating that it was derived from a different part of the enzyme.

A third TNP-peptide pool, fraction II from Sephadex G50 (Scheme I), was not adsorbed by CM-cellulose but was resolved into four TNP-peptide fractions on DEAE-cellulose. They represented only a small proportion of the original TNP content and were not studied further.

Discussion

Limited trinitrophenylation of ADP-glucose synthetase under the conditions of this study affected both the catalytic activity and structure of the enzyme. Evidence from several kinds of experiments indicated that a primary effect of trinitrophenylation was on the region of the enzyme essential for its normal allosteric regulation.

Table VI: Amino Acid Composition of the TNP-peptide Compared with the Allosteric Activator Site Pyridoxal-P Peptide[a]

	TNP-peptide A	TNP-peptide B	pyridoxal-P peptide[b]
alanine	4.2 (4)	5.9 (6)	(6)
arginine	4.4 (4)	6.9 (7)	(7)
aspartic acid	4.9 (5)	7.5 (7)	(5)
glutamic acid	4.9 (5)	5.1 (5)	(2)
glycine	3.5 (3)	6.2 (6)	(6)
histidine	1.2 (1)	1.5 (1)	(1)
isoleucine	2.9 (3)	4.2 (4)	(5)
leucine	3.8 (4)	6.6 (7)	(8)
lysine	2.0 (2)	3.0 (3)	(4)
methionine	0.1 (0)	0.1 (0)	(0)
phenylalanine	1.0 (1)	3.0 (3)	(3)
proline	1.4 (1)	2.5 (2)	(2)
serine	2.9 (3)	4.4 (4)	(3)
threonine	2.0 (2)	2.2 (2)	(2)
tyrosine	3.6 (4)	1.4 (1)	(0)
valine	3.3 (3)	5.4 (5)	(2)
homoserine[c]	0.6 (1)	0.8 (1)	(1)
pyridoxyllysine			(1)
TNP-lysine[d]	0.1	0.7	
tryptophan			(0)
cysteine			(1)

[a] The composition of TNP-peptide A and TNP-peptide B represents the average of two and three determinations, respectively, normalized to phenylalanine. The nearest integral values for residues per mole are expressed in parentheses. [b] Data from Parsons & Preiss (1978b). [c] The homoserine value was the sum of free homoserine and homoserine lactone. [d] The TNP-lysine content was determined spectrophotometrically before hydrolysis.

The major type of residue being modified was lysine. Of some 20 lysines present per subunit (Haugen et al., 1976a; Carlson et al., 1976), only about two appeared to be highly reactive with TNBS. The modification was highly specific, since a determination of the reaction order indicated that the reaction of only one molecule of TNBS per active unit of enzyme was sufficient for the complete loss of either synthesis or pyrophosphorolysis activity. Titration of the amino-terminal valine residue with FDNB suggested that there may also have been a low rate of reaction of it with TNBS.

Pyrophosphorolysis activity decreased about 3 times more slowly than synthesis. Also, when measured directly, two trinitrophenyl groups, rather than one, were incorporated accompanying complete inactivation of pyrophosphorolysis. Pyrophosphorolysis is normally stimulated about 2-fold (V_{max}) by the presence of the activator fructose-P_2, while synthesis is stimulated 6–7-fold. Thus, the striking difference in the response of the activity in the forward and reverse catalytic directions reflected interference with the ability of fructose-P_2 to function as activator. This was strongly supported by the finding that neither activity was much affected by trinitrophenylation when assayed under optimal conditions in the absence of fructose-P_2.

One of the effects of trinitrophenylation was to decrease the apparent affinity of the enzyme for either ATP or ADP-glucose, the first of the two ordered substrates to bind in each of the catalytic directions (Paule & Preiss, 1971). This would act in opposition to stimulation by the activator, as discussed above. The fact that subunit cooperativity in substrate saturation was not lost, while it was greatly reduced for activation, suggested that the effect of modification was localized to specific regions of one or more subunits, rather than for instance causing complete dissociation of the native enzyme tetramer to monomers. A direct examination of the molecular size of the modified enzyme confirmed that it did not dissociate upon trinitrophenylation.

The presence of substrates reduced trinitrophenylation and partially protected against loss of activity. The substrates were all somewhat less effective than the allosteric effectors as protective agents. The most efficient substrates at a given concentration were ATP and ADP-glucose. This was consistent with the sequential mechanism where either one can bind to the enzyme alone as the first substrate for the catalysis of synthesis or pyrophosphorolysis, respectively (Paule & Preiss, 1971). The binding of glucose-1-P or PP_i is efficient only in the presence of the nucleotide substrate (Haugen & Preiss, 1979). We attribute the protective effect of the substrates to the synergism among the catalytic and regulatory sites which has been shown by both kinetic and binding studies. Fructose-P_2 and either nucleotide substrate synergistically promote the binding of each other. ATP promotes the binding of AMP in the absence, but not in the presence, of fructose-P_2 (Haugen & Preiss, 1979). There is also strong interaction between activator and inhibitor sites. The total number of moles of hexanediol-P_2 plus AMP bound per tetramer does not exceed 4 (Haugen & Preiss, 1979). Nevertheless, the presence of a low concentration of fructose-P_2 enhances the inhibition by AMP (Gentner & Preiss, 1967). It is thus evident that there is a high degree of interaction between the binding sites for substrates and effectors. There are a number of ways this interaction can be altered, as illustrated by the studies on ADP-glucose synthetase from several mutant strains of *E. coli* B (Preiss, 1969, 1973) and from numerous species of photosynthetic and enteric bacteria (Preiss, 1978). The enzyme from a number of these is regulated by fructose-P_2, or AMP, or both. However, the enzymatic forms differ in their response to these effectors and in the interaction of effectors with each other and with substrates. Thus, it is not surprising to find here that limited chemical modification of the native enzyme by TNBS can simultaneously interfere with both fructose-P_2 activation and AMP inhibition.

One of the *E. coli* mutants mentioned above, SG5-504, yielded enzyme with normal affinity for substrates but altered in response to allosteric regulation. Structural evidence, including amino acid composition, subunit molecular weight, and a partial sequence, indicated that the alteration represented in the mutant enzyme was probably at the level of a single amino acid substitution (Carlson et al., 1976). The mutant differed from wild-type enzyme in its reaction with TNBS, under conditions similar to this study (Carlson et al., 1976). Since the present evidence suggests that the primary effects of trinitrophenylation are in the region of the allosteric site, a residue being trinitrophenylated and the mutation-related residue may be in the same vicinity. TNBS might therefore be useful in locating the alteration in the amino acid sequence of the mutant enzyme.

There is apparently more than one reactive lysine in, or at least directly affecting, the activator binding site. Thus, even though trinitrophenylation affected both fructose-P_2 activation and fructose-P_2-induced oligomerization, there was a lack of a significant effect on either the binding of or the activation by pyridoxal-P. The major target of trinitrophenylation was in the area of the amino terminus of the enzyme. The large isolated peptide (TNP-peptide B) contained a number of residues with positively charged side chains, including some four to five lysines, offering potential sites for attack by amino-modifying reagents. It was one of these same lysines that was preferentially reactive with pyridoxal-P (Parsons & Preiss, 1978b). In contrast, TNBS was not predominantly directed toward any one lysine. Instead, there was a distribution of the trinitrophenyl label among several lysines in TNP-peptide B, evidenced by a number of small TNP-peptides being generated by trypsin.

Such discrimination by two lysine-modifying reagents does have precedent. It has been observed that TNBS and pyridoxal-P react preferentially with different lysines in glutamate dehydrogenase (Coffee et al., 1971). Pyridoxal-P stoichiometrically modifies lysine-97, accompanying loss of catalytic activity (Piszkiewicz et al., 1970), while TNBS reacts primarily with lysine-425 and lysine-428 (Coffee et al., 1971). Trinitrophenylation affects several properties of the enzyme, but little loss of activity occurs (Goldin & Frieden, 1971).

Besides the residues affecting the allosteric activator site, there was reaction of TNBS with one or more residues apparently not directly involved in either catalysis or regulation, perhaps lying in TNP-peptide A.

It was evident that the extent of modification of the enzyme as originally measured spectrophotometrically was potentially misleading. Although 1.1 trinitrophenyl groups per subunit were incorporated in the enzyme used for the peptide study, this was in fact the net result of modification of several residues. It may be that the normal allosteric properties of the enzyme can be affected by the modification of one of a number of residues contributing positively charged side chains. As early as 1962, Hirs proposed that the particular reactivity of lysine-41 in ribonuclease was due to other nearby cationic groups, which together effectively reduced the pK of this ϵ-amino group and constituted an anion-binding site (Hirs, 1962). A similar effect in ADP-gluclose synthetase could explain the apparently equivalent reactivity of these several lysines for TNBS. One of the lysines of each subunit may be being independently modified, with the resulting additive disruption of allosteric activation. By contrast, pyridoxal-P is itself an effective allosteric activator of the enzyme and is thus directed by its structure to react with a specific lysine that is critical to the activation.

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Resonance Raman Spectra of Cytochromes *c* and *b* in *Paracoccus denitrificans* Membranes: Evidence for Heme–Heme Interactions†

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ABSTRACT: Resonance Raman (RR) scattering from cytochromes *b* and *c* in the bacterium *Paracoccus denitrificans* was recorded by exciting with the 568.2-, 530.9-, and 520.8-nm lines of a Kr^+ laser. The main features of the spectra were similar to those of the analogous cytochrome b–c_1 complex derived from pigeon breast mitochondria. Differences in the 1300-cm^{-1} region were interpreted in terms of marker bands for heme type and spectral coupling of the hemes on the membranes. It is difficult to explain the results without invoking sharing of electronic and vibrational wave functions among the hemes. This conclusion documents the potential to study the physics of electron transport in functioning membrane by monitoring the RR spectra.

Since the first reports of the resonance-enhanced Raman spectra of porphyrin in hemoglobin and cytochrome *c* (Brunner et al., 1972; Strekas & Spiro, 1972a,b), resonance Raman (RR) spectroscopy has been developed as a structural and dynamic probe of heme proteins. Rigorous descriptions of the scattering phenomenon in cytochrome *c* and other metalloporphyrins illustrated the elegant spectroscopic effects that this class of materials exhibits (Brunner et al., 1972; Collins et al., 1973; Friedman & Hochstrasser, 1973, 1976; Nafie et al., 1973; Verma et al., 1974; Woodruff et al., 1975; Asher & Sauer, 1976; Shelnutt et al., 1976, 1977; Kitagawa et al., 1976; Spiro & Loehr, 1975). Kinetic experiments on the nanosecond and picosecond time scale have documented differences in the dynamics of rebinding oxygen to hemoglobin and myoglobin following photolysis (Friedman & Lyons, 1980; Terner et al., 1980). More recently, a RR study of flavo-

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