Articles

Formation of an Intramolecular Cystine Disulfide during the Reaction of 8-Azidoguanosine 5′-Triphosphate with Cytosolic Phosphoenolpyruvate Carboxykinase (GTP) Causes Inactivation without Photolabeling†

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ABSTRACT: Phosphoenolpyruvate carboxykinase (GTP) (PEPCK) specifically utilizes a guanosine or inosine nucleotide as a substrate, yet it does not share extended sequence homology with other GTP-binding proteins, and the molecular basis for its nucleotide specificity is not understood. In an effort to locate the enzyme's nucleotide-binding site, we have studied the interaction of cytosolic PEPCK from rat liver with the photoprobe 8-azidoGTP, which fulfills the criteria of a specific photoaffinity label for PEPCK. The photoprobe binds reversibly to the enzyme prior to modification and at low concentrations causes greater than 60% inactivation (K_i = 1.2 μM). GTP provides nearly complete protection against inactivation by 8-azidoGTP, whereas phosphoenolpyruvate and metal ions provide partial protection. In addition, the photoprobe is a substrate for the enzyme and has a K_m similar to that for GTP. However, the extent of covalent modification by [^{32}P]8-azidoGTP as measured by three independent techniques is significantly lower than the extent of enzyme inactivation. Further investigation of this anomaly has revealed that the loss in enzymatic activity is caused by modification of a critical cysteine residue in a reaction that does not terminate with covalent attachment of the photolabel. Quantitation of the total free thiols of modified PEPCK shows that 2 mol of cysteine is lost per mole of inactivated enzyme. These results indicate that the photoinactivation of PEPCK by 8-azidoGTP is caused by the formation of an intramolecular cystine disulfide bridge, thus providing evidence for the existence of a pair of proximal cysteine residues within the GTP-binding site. The interaction of cysteine residues with the reactive photogenerated derivatives of 8-azidopurines is discussed.

Mammalian phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32), referred to hereafter as PEPCK,[1] exhibits a specific substrate requirement for guanosine or inosine nucleotides while catalyzing the first committed step of gluconeogenesis:

oxaloacetate + MgGTP (ITP) ⇌ phosphoenolpyruvate + MgGDP (IDP) + CO_2

Adenosine nucleotides are neither substrates nor inhibitors and do not bind detectably to the enzyme (Miller et al., 1968). The gene coding for the cytosolic carboxykinase has recently been cloned, and the amino acid sequence has been deduced and confirmed by fast atom bombardment mass spectroscopy (Beale et al., 1985). Although the PEPCK sequence does not share extensive regions of homology with other GTP-binding proteins, it does contain three consensus sequences that are believed to represent the guanine nucleotide binding region (Cook et al., 1986). To date, however, the nucleotide-binding site of PEPCK has not been identified, and very little is known about the structure of the enzyme's active site or about the chemical basis for its nucleotide specificity.

We have recently identified a very reactive cysteine residue of PEPCK (Cys-288) that is essential[2] for catalytic activity and that lies between two putative phosphoryl-binding regions (Lewis et al., 1989; Carlson et al., 1978). Although the enzyme contains 13 cysteine residues (all in the reduced state) among its 622 amino acids (Carlson et al., 1978; Beale et al., 1985),[3] Cys-288 can be rapidly and exclusively modified by incubation of the enzyme with equimolar levels of a sulfhydryl reagent such as a hydrophobic maleimide. Nucleotides provide almost complete protection against modification of this cysteine, suggesting that it may reside within or near the enzyme's active site (Lewis et al., 1989). There is considerable evidence that Cys-288 exists in a vicinal thiol pairing with another

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[1] Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase (GTP); PEP, phosphoenolpyruvate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; 8-N_3GTP, 8-azidoguanosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate.

[2] By "essential", we refer to data that indicate that the apparently exclusive modification of Cys-288 causes a nearly complete loss of all catalytic activities of PEPCK. However, solely on the basis of chemical modification experiments, it is difficult to classify any residue as being truly essential, i.e., absolutely necessary for expression of any catalytic activity.

[3] The residue numbering system used herein is based upon 622 amino acids and includes the amino-terminal methionine as residue 1. It is not clear if the N-terminal Met is cleaved or modified in vivo, because its presence has not been confirmed by amino-terminal sequencing (Beale et al., 1985).

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cysteine, but the identity of the proximal cysteine has not yet been determined. (Vicinal is used herein to denote spatially proximal residues as opposed to residues that are adjacent in the sequence.) These two proximal cysteines can be induced to form a cystine disulfide by incubation of PEPCK with equimolar Nbs_2 in the absence of substrates; under these conditions 2.0 mol of mercaptonitrobenzoate is released per mole of enzyme, and the loss of catalytic activity is nearly complete. Nucleotide, or a combination of metal and nucleotide, can provide substantial protection not only against disulfide bridge formation but also against total thiol modification by an excess of sulfhydryl reagent (Carlson et al., 1978). Thus, numerous results suggest that 1 or more of the enzyme's 13 cysteines reside within or near the GTP binding site.

We have employed the photoaffinity label 8-N_3GTP in an attempt to locate the nucleotide-binding site of PEPCK by isolation of a modified peptide. We have shown that 8-N_3GTP fulfills all of the criteria of a photoaffinity label for this enzyme and is capable of causing a large amount of enzyme inactivation at very low inhibitor concentrations. However, our data indicate that the photoprobe undergoes an irradiation-dependent reaction at the enzyme's active site that causes formation of a cystine disulfide, resulting in a substantial loss of catalytic activity without incorporation of radioactive label.

Experimental Procedures

Materials. 8-N_3GTP, [γ-^{32}P]8-N_3GTP, and [α-^{32}P]8-N_3GTP were synthesized as previously described (Geahlen & Haley, 1977) and stored in methanol in the dark at −80 °C. The purity of aqueous solutions of nonradioactive and carrier-free [^{32}P]azidoGTP was routinely monitored by thin-layer chromatography (Czarnecki et al., 1979) and by ultraviolet absorption spectroscopy (Geahlen & Haley, 1977) in order to assess the extent of contamination by 8-N_3GDP and the extent of reduction of the azido moiety, respectively. Because radioactive [^{32}P]azidoGTP is especially sensitive to spontaneous reduction to form a nonreactive species, the radioactive photoprobe was always used within a short time after synthesis and under conditions in which the major species was the photoactivatable 8-N_3GTP. The concentration of 8-N_3GTP was determined spectrophotometrically from an extinction coefficient of 12 000 M^{-1} cm^{-1} at 278 nm (Geahlen & Haley, 1977). Reduced 8-azidoGTP (8-aminoGTP) was prepared by incubating 8-N_3GTP with excess dithiothreitol for 5 h at 5 °C. In contrast to 8-N_3GTP, the absorption spectrum of 8-aminoGTP was unchanged following extensive irradiation of the sample, indicating that the reduction of 8-N_3GTP had proceeded to completion (Potter & Haley, 1983). The 8-aminoGTP was purified by chromatography over a Bio-Gel P-2 column (1 × 25 cm) equilibrated in 10 mM ammonium bicarbonate, pH 7.5; the complete removal of dithiothreitol from the nucleotide was verified by assaying each column fraction for thiols with Nbs_2.

PEPCK from rat liver cytosol was purified to homogeneity and to a high, constant specific activity (18–23 μmol of oxaloacetate formed per minute per milligram at 25 °C) as previously described (Colombo et al., 1978; Lewis et al., 1989). Exogenous thiols were removed by gel filtration in the presence of 10 mM TES (pH 7.2), 5% glycerol, and 0.5 mM EDTA, and the concentration of PEPCK was determined spectrophotometrically from a molar extinction coefficient[4] of 1.15 × 10^5. All buffers were degassed and saturated with nitrogen. The PEPCK-catalyzed rates of phosphoenolpyruvate formation or oxaloacetate formation were determined at 25 °C as described previously (Colombo et al., 1978; Lewis et al., 1989).

Standard Conditions for the Reaction of PEPCK with 8-N_3GTP. In preliminary experiments, standard conditions for photolabeling were determined such that a minimal duration of irradiation caused a maximal level of 8-N_3GTP-dependent enzyme inactivation. Control experiments showed that the chemical integrity of 8-N_3GTP, as assessed by ultraviolet absorption spectroscopy and thin-layer chromatography, was not affected by incubation with any of the buffer components or PEPCK. The photoinactivation was performed at 0 °C in a glass well in a total volume of 50 μL. PEPCK (0.1–5 μM) was incubated with 8-N_3GTP or [^{32}P]8-N_3GTP and various effectors in the presence of 33 mM HEPES, 6 mM TES (carried over with enzyme), 3% (v/v) glycerol, and 300 μM EDTA at pH 7.2. Each sample was irradiated at 0 °C with a UVS-11 Mineralight lamp (254 nm) at a distance of 1 cm. The optimal duration of irradiation necessary for maximal photolysis of 8-N_3GTP and minimal inactivation of PEPCK alone was determined to be 25 s; at higher enzyme concentrations the samples were irradiated for 45 s. After irradiation, an aliquot of the reaction mixture was assayed at 25 °C with the appropriate coupled continuous spectrophotometric assay for phosphoenolpyruvate carboxylation or oxaloacetate decarboxylation activity (Colombo et al., 1978).

The extent of enzyme inactivation was always assessed relative to that of control enzyme that was irradiated in the absence of 8-N_3GTP; the loss in PEPCK activity due solely to irradiation was consistently less than 10%. The inactivation of PEPCK by 8-N_3GTP was not due to an effect on the coupling enzymes used in the continuous spectrophotometric assays because neither prephotolyzed nor nonphotolyzed 8-N_3GTP had any effect on the activities of malate dehydrogenase, pyruvate kinase, or lactate dehydrogenase when included in these assays at concentrations exceeding those used to photoinactivate PEPCK. Preincubation of PEPCK with 8-N_3GTP for various lengths of time prior to irradiation did not cause any significant difference in the extent of inactivation; therefore, the reaction mixture was not preincubated but was simply mixed and immediately irradiated.

Measurement of Photoincorporation of [^{32}P]8-N_3GTP. The incorporation of [α-^{32}P]- or [γ-^{32}P]8-N_3GTP into the enzyme was determined by three independent methods: (1) acid precipitation onto filter paper (Reimann et al., 1971; King et al., 1982), (2) polyacrylamide gel electrophoresis, and (3) gel filtration under denaturing conditions. Electrophoresis on 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate was performed as described by Laemmli (1970). The labeled enzyme was identified by staining with Coomassie blue or by autoradiography of the unstained gel. The bands were excised and solubilized by heating with 0.3 mL of 30% H_2O_2 for 3 h at 80 °C. The ^{32}P content of each sample was determined by liquid scintillation counting after the addition of 4 mL of Aquasol (Du Pont) scintillation fluid. Gel filtration of the labeled enzyme was performed by combining aliquots from several reaction mixtures and adding urea to a final concentration of 6 M. Each sample (approximately 350 μL) was passed over a 10-mL column (0.7 × 26 cm) of Sephadex G-50 equilibrated in 50 mM ammonium bicarbonate (pH 8.5) and 6 M urea. Under these conditions the labeled enzyme (void volume) was completely separated from free [^{32}P]8-N_3GTP. Each collected fraction (420 μL) was assayed for ^{32}P by liquid scintillation counting and for protein with the

[4] The molar extinction coefficient was based on a previous determination and was corrected for the exact molecular weight of phosphoenolpyruvate carboxykinase (Beale et al., 1985; Colombo et al., 1978).

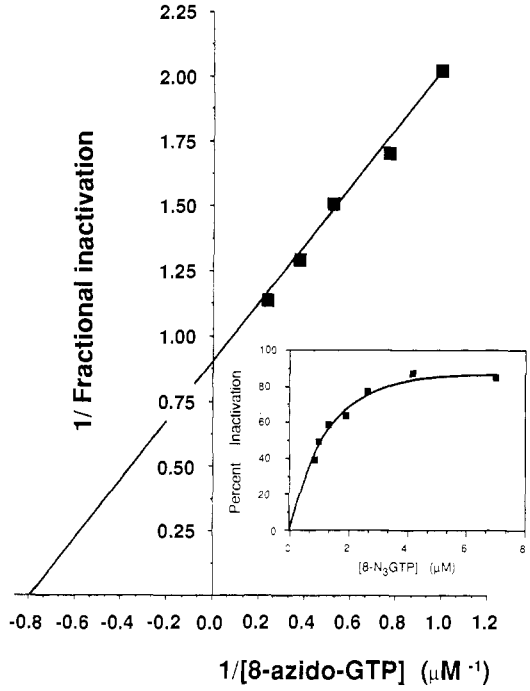


FIGURE 1: Inactivation of PEPCK by 8-N_3GTP. PEPCK (0.1 μM) was incubated at 0 °C with the indicated concentrations of 8-N_3GTP and photolabeled under standard conditions as described under Experimental Procedures. After 25 s of irradiation, 45-μL aliquots were diluted 22-fold, and the rate of phosphoenolpyruvate formation was determined. The figure represents a double-reciprocal plot of the data shown in the inset. Inset: Dependence of inactivation of PEPCK on 8-N_3GTP concentration.

Bio-Rad protein assay reagent; unmodified PEPCK in the presence of an equivalent concentration of urea was used as the protein standard.

Thiol Titration of Native and Modified PEPCK. The total cysteine content of native and modified PEPCK was assessed by titration of the free thiols with excess Nbs_2 in the presence of 1% sodium dodecyl sulfate. For these experiments, the photoinactivation was performed in a quartz cuvette at 25 °C. In a total volume of 550 μL of buffer [100 mM TES (pH 7.0), 5% glycerol, 0.5 mM EDTA], PEPCK (3–8 μM) was irradiated in the presence or absence of different concentrations of 8-N_3GTP at a distance of 1 cm for 45 s. After aliquots of the reaction mixture were assayed to determine the extent of inactivation, the mixture was brought to 1% sodium dodecyl sulfate, and the titration was initiated with 1.2 mM (final concentration) Nbs_2. The absorbance at 412 nm was monitored with time on a Beckman DU-70 spectrophotometer and was measured against a blank that contained all components of the reaction except enzyme. The extinction coefficient used for the mercaptonitrobenzoate anion was 14150 M^{-1} cm^{-1} (Riddles et al., 1983). All results, with respect to both inactivation and total thiol content, were assessed relative to control enzyme that was irradiated in the absence of 8-N_3GTP. In each experiment, the control enzyme had the expected 13 thiols.

Results

Behavior of 8-N_3GTP as a Specific Photoaffinity Label for PEPCK. The identification of an active site residue by modification with a photoactivatable substrate analogue first requires that the specificity of the reaction be firmly established. We thus asked whether 8-N_3GTP could fulfill the criteria of a specific affinity label for PEPCK. Figure 1 shows the dependence of PEPCK inactivation on the concentration of 8-N_3GTP (inset) and the double-reciprocal plot derived from these data. The saturation in the extent of enzyme inactivation with increasing concentrations of 8-N_3GTP indicates that the photoprobe binds reversibly to PEPCK prior to photolysis-dependent inactivation. The K_i determined from the intercept of the double-reciprocal plot was 1.23 ± 0.06 μM (mean ± SD, n = 2). The data in Figure 1 were obtained under pseudo-first-order conditions, in which the PEPCK concentration was necessarily low and the ratio of [8-N_3GTP] to [PEPCK] exceeded 10:1; however, the concentration dependence of inactivation by 8-N_3GTP was similar if the PEPCK concentration was of the same order of magnitude as that of the photoprobe, a condition used in subsequent experiments to measure substrate protection. The large extent of enzyme inactivation caused by low concentrations of photoprobe suggests that significant quantities of modified enzyme could be obtained without sacrificing the specificity of labeling. Photoinactivation by 8-N_3GTP caused similar losses in the abilities of PEPCK to catalyze the formation of PEP (forward reaction) or oxaloacetate (reverse reaction) (data not shown).

It should be emphasized that the inactivation of PEPCK that we observed was not due to destruction of the enzyme by the ultraviolet irradiation alone, because all results were assessed relative to control enzyme that was irradiated in the absence of 8-N_3GTP. Several studies have shown a loss in activity of a probe's receptor during photoaffinity labeling to be caused not by covalent modification but by the tight, noncovalent binding to the receptor of photolytic breakdown products of the probe (Fisher & Press, 1974; Standring & Knowles, 1980); however, this was not the cause of the inactivation of PEPCK by 8-N_3GTP, because prephotolyzed 8-N_3GTP had no effect on PEPCK activity when included in assays at concentrations greater than or equal to those used in the photoaffinity labeling experiments. Moreover, prephotolysis of 8-N_3GTP for 25 s followed by the immediate addition of PEPCK resulted in no inactivation during a subsequent 5-min incubation, which indicates that the inactivation observed under standard photolysis conditions is not due to the action of a long-lived, photogenerated reactive intermediate of 8-N_3GTP. Other evidence against such an intermediate is that the addition to the photolysis mixture of 10 mM Tris or *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) as scavenger had no significant effect on the ability of 8-N_3GTP to inactivate PEPCK. It is also unlikely that the enzyme inactivation is caused simply by a ligand- and irradiation-dependent, but azide-independent, oxidation reaction (i.e., ligand-sensitized photooxidation) because there is no loss in the activity of enzyme when irradiated in the presence of 8-aminoGTP or GTP under standard photolysis conditions; in fact, GTP appears to protect against irradiative damage to PEPCK (Lewis and Carlson, unpublished observations).

If an affinity label modifies the active site of an enzyme, then the true substrate should provide specific protection against inactivation. Figure 2 shows that this was indeed the case; even at low GTP concentrations the photoinactivation was substantially reduced. Furthermore, the protective effect exerted by GTP was due to specific substrate protection and not merely to a decreased efficiency of photolabeling caused by absorption of the ultraviolet light by the nucleotide, because AMP, which is not a substrate, provided insignificant protection against the inactivation. Although metal-chelated nucleotide is required for catalysis by PEPCK of either the forward or reverse reactions, free GTP is capable of binding rather tightly to the enzyme. [The K_d is 3.4 μM for the binding of free GTP to PEPCK from hog liver mitochondria (Miller et al., 1968).] The abilities of other substrates to

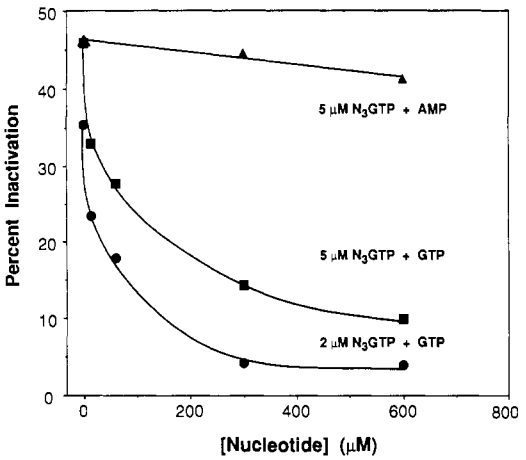


FIGURE 2: Protection by GTP against enzyme inactivation. Photolabeling was performed under standard conditions with 1 μM PEPCK, 5 μM 8-N_3GTP (▲, ■) or 2 μM 8-N_3GTP (●), and the indicated concentrations of GTP (■, ●) or AMP (▲). After 25 s of irradiation, aliquots of the reaction mixture were diluted 33-fold, and the rate of oxaloacetate formation was determined. The control enzyme was treated identically (±GTP or ±AMP), but in the absence of 8-N_3GTP.

Table I: Substrate Protection against Inactivation by 8-N_3GTP[a]

	addition	% inactivation
A	none	60.8 ± 0.2
	2 mM phosphoenolpyruvate	31.4 ± 2.0
	2 mM HCO_3^-	64.3 ± 1.2
	2.25 mM $Mg(CH_3COO)_2$	28.8 ± 0.6
	2.25 mM $MnCl_2$	27.7 ± 1.9
	2.25 mM $Mg(CH_3COO)_2$ + 120 μM $MnCl_2$	30.0 ± 2.3
B	none	66.3 ± 1.4
	300 μM GTP	13.0 ± 3.9
	300 μM oxaloacetate	63.1 ± 0.8
	4 mM $Mg(CH_3COO)_2$	27.8 ± 2.7

[a] The photoinactivation of PEPCK by 8-N_3GTP was carried out under standard conditions in the presence of 1 μM PEPCK, 5 μM 8-N_3GTP, and the indicated concentrations of substrates. Aliquots of the reaction mixture were diluted 33-fold, and the rates of oxaloacetate formation (A) or PEP formation (B) were measured. In each case control enzyme was treated identically, but in the absence of 8-N_3GTP; the results are an average of duplicate experiments (mean ± SD).

protect against photoinactivation by 8-N_3GTP are illustrated in Table I. GTP provided the most effective protection against inactivation, whereas PEP provided partial protection and oxaloacetate and bicarbonate were relatively ineffective. The addition of metal ions caused a substantial decrease in the extent of inactivation. Unlike Mg^{2+}, free Mn^{2+} is an activating divalent cation for PEPCK—inclusion of micromolar levels of Mn^{2+} in the PEPCK assays stimulates the rate of PEP production and is nearly obligatory for oxaloacetate production (Colombo et al., 1981; Colombo & Lardy, 1981). If productive occupancy of the free metal binding site were to influence the inactivation of PEPCK by 8-N_3GTP, one would expect to observe different levels of enzyme inactivation in the presence of Mg^{2+} (which does not activate PEPCK), Mn^{2+}, or Mg^{2+} plus Mn^{2+}; however, this was not observed. Because either divalent cation provided almost identical protection, it seems more likely that their action is exerted through a common mechanism, such as through chelation of the 8-N_3GTP (perhaps to increase specificity) or through a general cation effect.

An additional criterion for a compound that behaves as an affinity label is catalytic competence. Our results showed that 8-N_3GTP is a substrate for PEPCK in the assay that measures the rate of PEP formation, thus fulfilling this criterion (Figure 3). The apparent K_m for 8-N_3GTP was 36.6 μM, compared to the $K_{m,app}$ for GTP of 17.6 μM; the V_{max} was virtually

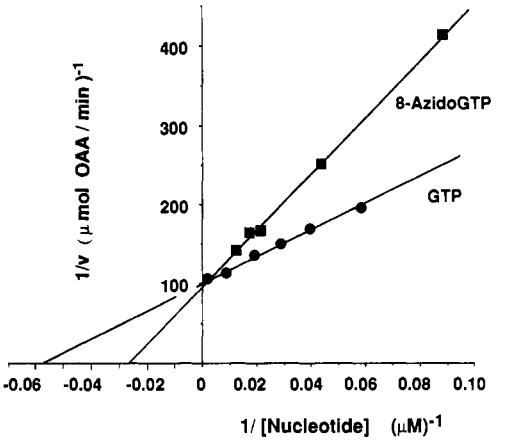


FIGURE 3: Behavior of 8-N_3GTP as a substrate for PEPCK. The rate of phosphoenolpyruvate formation was measured at 25 °C in the presence of GTP (●) or 8-N_3GTP (■) as described under Experimental Procedures. A double-reciprocal plot of the data is presented. OAA refers to oxaloacetate.

identical for both compounds.

Comparison of Photolabeling with Photoinactivation. The specificity of a photolabel can be further defined by establishing a strict correlation between incorporation of the probe and loss in activity of the probe's receptor. The covalent incorporation of radioactive 8-N_3GTP into PEPCK was measured by three independent methods, as described under Experimental Procedures. In the first method, the enzyme was precipitated onto filter paper in the presence of cold 10% trichloroacetic acid; the filter paper was washed to remove noncovalently bound [^{32}P]8-N_3GTP, and the radioactivity was quantitated. With this method, we were unable to detect significant incorporation of either [α-^{32}P]- or [γ-^{32}P]8-N_3GTP into PEPCK (less than 0.01 mol of 8-N_3GTP/mol of enzyme), in spite of the fact that greater than 50% of the enzyme had been inactivated. Similar results were obtained when albumin was added as a carrier protein to an aliquot of the reaction mixture prior to the acid precipitation. The photoincorporation of [^{32}P]8-N_3GTP as measured by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was also very low; again, less than 0.01 mol of [α-^{32}P]- or [γ-^{32}P]8-N_3GTP was incorporated per mole of enzyme. The very low extent of incorporation was obtained whether or not the gels were stained and destained prior to solubilization and counting and whether or not 10% β-mercaptoethanol was included in the buffer used to treat the samples for gel electrophoresis [125 mM Tris (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol]. The low photoincorporation as measured by these two techniques was also independent of a variety of reaction conditions: substitution of phosphate or TES buffer for HEPES or alteration of pH or ionic strength. Increasing the duration of irradiation had no effect on the photoincorporation, and performing repetitive 25-s irradiations, with fresh additions of [^{32}P]8-N_3GTP before each irradiation, caused only a slight increase in the incorporation of label.

Significant amounts of covalently modified enzyme could be detected only under the less harsh conditions of gel filtration in the presence of denaturant. For these experiments, photoinactivated PEPCK was denatured by the addition of urea (to 6 M) and was separated from noncovalently bound [^{32}P]8-N_3GTP by desalting in the presence of 50 mM ammonium bicarbonate (pH 8.5) and 6 M urea. Determination of protein and ^{32}P in each fraction indicated that 0.2 mol of [^{32}P]8-N_3GTP was incorporated per mole of PEPCK (0.35 mol of [^{32}P]8-N_3GTP/mol of inactivated enzyme). A similar extent of labeling was observed if the 6 M urea was replaced

by 0.1% or 1% sodium dodecyl sulfate. The low extent of modification that we could detect by any of the three methods was apparently specific, because GTP provided almost complete protection against inactivation and photolabeling.

Elimination of Possible Explanations for the Lack of Correlation between Labeling and Inactivation. Although the labeling appeared to be specific and although the stoichiometry of labeling by [^{32}P]8-N_3GTP as measured by gel filtration was at least 10-fold higher than that detected under more harsh assay conditions, the photoincorporation was nevertheless substantially lower than the extent of enzyme inactivation. We were able to rule out several possible explanations for the lack of correlation between photoincorporation and photoinactivation. (1) The enzyme inactivation was not simply due to ultraviolet irradiation, as discussed previously. (2) The low labeling was not due to poor chemical integrity of only the radioactive 8-N_3GTP. If the radioactive 8-N_3GTP were reduced and therefore not photoactive while the carrier nonradioactive 8-N_3GTP were of high chemical integrity, one would expect to observe loss in enzyme activity with little or no incorporation of radioactive probe. However, the integrity of carrier-free [α-^{32}P]- and [γ-^{32}P]8-N_3GTP was monitored prior to each experiment, and the absorption spectra indicated that the predominant species was the photoactivatable azidoGTP. (3) In some instances, the photolytic breakdown products of a photoaffinity label can bind more tightly to a receptor than the photoprobe itself; if this resulted in exclusion of the reactive photogenerated derivative, one would again predict that the loss of catalytic activity would exceed the covalent modification. However, as was discussed previously, our results indicated that the photolytic breakdown products of 8-N_3GTP did not have a significant effect on PEPCK activity. (4) The low incorporation was not caused by a loss of the ^{32}P label upon chemical or enzymatic hydrolysis of the enzyme-bound azidoGTP. Although thin-layer chromatography indicated that hydrolysis of the free 8-N_3GTP was negligible, we considered the possibility that a GTP derivative covalently attached within the enzyme's active site might undergo hydrolysis of the γ-phosphoryl group, which would result in inactivation but not incorporation of radioactivity. However, because the incorporation of [α-^{32}P]- and [γ-^{32}P]8-N_3GTP was equally low, this explanation was also eliminated.

Interaction of 8-N_3GTP with Enzyme Thiols. The results indicated that the disparity between inactivation and incorporation could not be explained by commonly encountered problems of photoaffinity labeling but rather appeared to be specific to our system. Our knowledge of the hyperreactive, essential cysteine led to the hypothesis that the inactivation and modification of PEPCK was caused by the formation of a labile bond between a critical cysteine and the reactive photogenerated derivative of 8-N_3GTP. On the assumption that such a modification might be reversed by excess dithiothreitol, this hypothesis was tested. Dilution of photoinactivated PEPCK in the presence of a large excess of dithiothreitol resulted in a nearly complete regain of PEPCK activity (Table II). The reactivation occurred rapidly, reaching completion within 2 min. However, dithiothreitol did not decrease the amount of photolabeling detected by gel filtration: the photoincorporation of [^{32}P]8-N_3GTP into PEPCK that had been nearly completely reactivated by dithiothreitol was identical with that incorporated into enzyme that had not been reactivated (treated identically but without subsequent addition of dithiothreitol) (data not shown). These results indicate that at least two modification events occur upon irradiation. First, a thiol residue is modified in a manner that does not result in detectable covalent attachment of the radioactive photoprobe; this modification causes the majority of the loss of enzymatic activity and is reversed by the addition of excess dithiothreitol. Second, a residue(s) is modified by covalent attachment of the radioactive 8-N_3GTP derivative through a linkage that is not affected by excess dithiothreitol; this modification represents most, if not all, of the detectable incorporation of radioactivity. It is difficult to assess the extent of enzyme inactivation caused by this dithiothreitol-insensitive modification, but it can only represent a small fraction of the total because of the nearly complete reactivation by dithiothreitol (Table II). Attempts to identify the residues modified by the dithiothreitol-insensitive labeling were not successful.

In order to prove that the inactivation of PEPCK by 8-N_3GTP is caused by the modification of a thiol residue, the total free thiols of enzyme photoinactivated to different extents (by varying the concentration of 8-N_3GTP) were quantitated by titration with Nbs_2 (Figure 4). The plot of percent residual activity versus moles of SH lost per mole of enzyme extrapolates to 1.81 at zero activity, indicating that the loss in enzyme activity is caused by the modification of two cysteine residues. Because approximately 2 mol of cysteine is lost per mole of inactivated enzyme and because this modification is not ac-

Table II: Reactivation by Dithiothreitol of Photoinactivated PEPCK[a]

treatment		
irradiation	dilution	specific activity (SD)[b]
-N_3GTP	-dithiothreitol	13.92 (0.02)[c]
+N_3GTP	-dithiothreitol	1.75 (0.06)
-N_3GTP	+dithiothreitol	14.71 (0.52)
+N_3GTP	+dithiothreitol	14.06 (0.25)

[a] PEPCK (1 μM) was irradiated in the presence or absence of 5 μM 8-N_3GTP for 25 s as described under Experimental Procedures. Aliquots of each reaction mixture were then diluted 20-fold into buffer (10 mM TES, pH 7.0, 5% glycerol, 0.5 mM EDTA) that did, or did not, contain dithiothreitol (10 mM). The specific activity (oxaloacetate formation) of PEPCK was measured after 2 min of incubation at 0 °C. [b] The results are expressed as the mean (SD) for duplicate experiments. [c] The specific activity of PEPCK that was irradiated in the absence of 8-N_3GTP was 19.2 units/mg, but control experiments indicated that the 20-fold dilution caused an approximate 25% loss in actvity at these low enzyme concentrations.

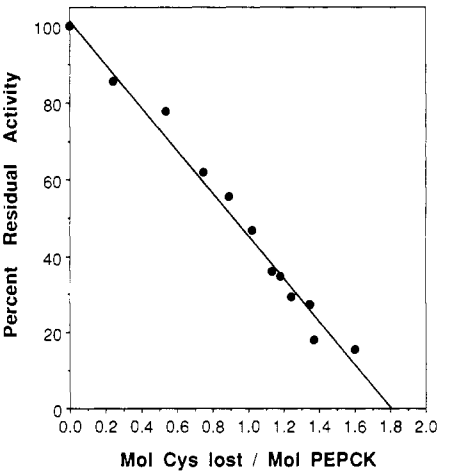
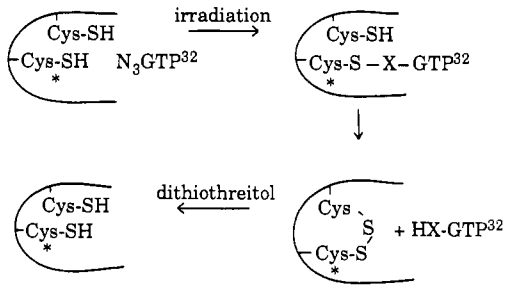


FIGURE 4: Correlation of inactivation with loss of thiols. PEPCK (3–8 μM) was irradiated in the presence of different concentrations of 8-N_3GTP in a quartz cuvette at a distance of 1 cm for 45 s. After the irradiation, aliquots of the reaction mixture were assayed to determine the loss of oxaloacetate-forming activity. The total cysteine content of each sample was then determined by spectrophotometric titration with Nbs_2 following addition of sodium dodecyl sulfate to each cuvette (final concentration 1.0%). The data represent the results of different experiments with different enzyme preparations.

Scheme I



companied by detectable incorporation of [^{32}P]8-N_3GTP (dithiothreitol reverses the inactivation but has no significant effect on the photolabeling), we conclude that the inactivation of PEPCK by 8-N_3GTP is caused by the formation of an intramolecular disulfide bridge.

Attempts to stabilize a cysteine-labeled intermediate by blocking disulfide formation were not successful; the addition of excess iodoacetate to the reaction mixture immediately after irradiation had no effect on the extent of labeling. Similarly, PEP, which is known to prevent formation of the cystine disulfide induced by Nbs_2 (Carlson et al., 1978), did not inhibit disulfide formation when added immediately after irradiation. Inclusion of PEP in the reaction mixture during irradiation protected against inactivation (Table I) and disulfide bridge formation but also protected against incorporation of [γ-^{32}P]8-N_3GTP. Attempts to trap the cysteine-linked photogenerated intermediate through displacement with [^{14}C]-cyanide also were unsuccessful. Studies are currently underway to directly identify the cysteine residues composing the disulfide that is formed during the reaction with 8-N_3GTP.

Discussion

Our results indicate that 8-N_3GTP is a GTP photoaffinity analogue for phosphoenolpyruvate carboxykinase. The photoprobe fulfills the criteria of an affinity label in that it is catalytically competent and is capable of providing substantial, concentration-dependent, saturable enzyme inactivation that is nearly completely prevented by inclusion of the true substrate. Furthermore, 8-N_3GTP exhibits a very low K_i that is similar to the K_d previously determined for GTP (Miller et al., 1968), thus permitting the use of low concentrations of photoprobe for modification of the enzyme. On the basis of all of our data, it is highly probable that the 8-N_3GTP-dependent loss in PEPCK activity reflects a modification event that occurs at or near the GTP-binding site.

The modification of PEPCK by the photoprobe can be described by three parameters: the extent of enzyme inactivation, the extent of covalent incorporation of radioactive label, and the effect of dithiothreitol. Consideration of each of these parameters has allowed us to dissect the modification events that occur. It is clear that most of the loss of PEPCK activity is caused by the modification of two cysteine residues, at least one of which is critical. Although dithiothreitol can nearly completely reverse the loss in enzymatic activity, it does not decrease the detectable labeling, suggesting that the dithiothreitol-reversible inactivation is not associated with detectable covalent attachment of radioactive photoprobe. The mechanism of photoinactivation that is consistent with all of the data involves the formation of an intramolecular cystine disulfide as shown in Scheme I. In this model, the 8-N_3GTP binds specifically within the enzyme's active site. Upon irradiation, the photogenerated derivative forms a transient intermediate (X-GTP^{32}) with a critical thiol (indicated by the asterisk), resulting in loss of activity. The intermediate is rapidly attacked by a nearby thiol, causing formation of an intramolecular cystine disulfide while maintaining loss of catalytic activity; this reaction is accompanied by the release of the radioactive derivative of 8-N_3GTP (HX-GTP) from the modified cysteine. The subsequent addition of dithiothreitol reduces the disulfide and results in the regain of PEPCK activity.

The data in Figure 4 and Table II indicate that the predominant inactivation event involves the loss of two cysteine residues through formation of a disulfide bridge. Although the data points extrapolate to a number that is slightly less than 2 mol of cysteine lost per mole of inactivated enzyme, there are at least two reasons why extrapolation to 2.0 should not be expected. First, other modification events, such as the dithiothreitol-insensitive labeling, may give rise to a small amount of inactivation independent of disulfide bridge formation. Second, the displacement of the photogenerated derivative by the vicinal thiol may not always proceed to 100% completion, which would also result in fewer than two cysteine residues modified per inactivation event.

There would seem to be three potential mechanisms that could lead to formation of a disulfide bond during the irradiation of PEPCK in the presence of 8-N_3GTP. Our data are most consistent with a mechanism in which formation of the intramolecular disulfide occurs through a transient, covalent intermediate between a reactive, photogenerated derivative of specifically bound 8-N_3GTP and an enzymatic thiol. This mechanism, shown in Scheme I, corresponds to typical affinity labeling. An alternative mechanism is that a relatively long-lived, photogenerated derivative of 8-N_3GTP might simply act as a general chemical modifier to attack the highly reactive, nucleophilic, essential cysteine (Cys-288) to form a transient, covalent intermediate; however, there are several reasons why modification by a long-lived, photogenerated derivative is inconsistent with our data. First, the immediate addition of PEPCK to prephotolyzed 8-N_3GTP did not result in inactivation. Second, the addition of scavengers during photolysis with 8-N_3GTP had no significant effect on inactivation. Third, inactivation was saturable with increasing concentrations of 8-N_3GTP, which indicates specific binding prior to modification. If the photogenerated derivative of 8-N_3GTP were acting as a general thiol reagent to modify a hyperreactive cysteine residue, one would not expect saturation with increasing concentrations of photoprobe. It is somewhat more difficult to eliminate the third potential mechanism for disulfide formation, which is that specifically bound 8-N_3GTP acts as a photosensitizer to promote disulfide formation through some type of photooxidation reaction; however, irradiation of PEPCK under standard conditions in the presence of 8-aminoGTP or GTP does not result in inactivation, even at high concentrations of nucleotide. Finally, the chemistry of the initial covalent bond that would likely be formed between a thiol and a photogenerated nitrene, or reactive derivative thereof, is entirely consistent with the eventual formation of a disulfide bond, as will be discussed.

Although it is often suggested that photogenerated reagents can react with nearly any amino acid side chain to form a stable covalent bond, aryl azides may, in fact, react preferentially with nucleophiles (Bayley & Knowles, 1978; Brunner & Richards, 1980), and the stability and character of the resultant linkages are rarely investigated. An S–N (sulfenamide) bond between a cysteine and an aryl nitrene could be generated simply by abstraction of a thiol hydrogen by the nitrene [Guillory and Jeng (1983) and references cited therein]; alternatively, the nitrene could rearrange before re-

acting with the nucleophile, as has been described for phenyl azides (Iddon et al., 1979; Bayley & Staros, 1984; Leyva et al., 1986).[5] The stability and reactivity of S–N bonds has been studied extensively because of the utility of sulfenamides as herbicides, fungicides, accelerators in the vulcanization of rubber, and intermediates in synthetic processes [for reviews, see Brown and Grayson (1970) and Davis (1973)]. The polarity of the sulfur–nitrogen bond makes sulfenamides susceptible to both nucleophilic and electrophilic attack; they are readily hydrolyzed in acidic solutions and are known to undergo facile cleavage by thiols (Kharasch et al., 1946). Free thiols react with sulfenamides (Armitage et al., 1972) and sulfenimides (Harpp et al., 1970) to form mixed-function disulfides. Therefore, on the basis of the known chemistry of sulfenamides, one would predict that an S–N bond formed by the reaction of cysteine and a photogenerated derivative of 8-N_3GTP would be acid labile and could undergo cleavage by a neighboring cysteinyl thiol to form a disulfide. The presence of spatially proximal cysteine residues known to exist in PEPCK would increase the probability of disulfide formation. If a vicinal thiol were not present to attack the S–N bond, the sulfenamide could alternatively by hydrolyzed during peptide isolation, which would also eliminate the photoprobe. Sulfenic acid, the likely initial product of hydrolysis,[6] could, in turn, have multiple fates (Allison, 1976; Davis et al., 1986).

There are only a few reports in the literature describing the modification of a cysteine residue by an aryl azide. Using microsequencing and fast atom bombardment mass spectroscopy, Chen et al. (1986) obtained direct evidence for the specific photolabeling of a cysteine residue of glyceraldehyde-3-phosphate dehydrogenase by arylazido-β-alanyl-NAD^+. A sulfenamide structure was proposed for the modified peptide, and further mass spectral analyses suggested that the sulfenamide bond was labile and could be broken in the presence of dithiothreitol. In a thorough study, Vandest et al. (1980) provided strong evidence for the highly specific, covalent labeling of cysteine residues of arginine and creatine kinases by ATP γ-(*p*-azidoanilide). The bond linking the photoprobe to the enzymes appeared to be stable under the experimental conditions tested (extensive dialysis at pH 7.5 and two-dimensional peptide mapping at pH 6.5); however, the photogenerated derivative that reacted with the cysteines in that study appears to be different from that generated from 8-N_3GTP in that the reactive azidoanilide derivative was very long-lived. Hoppe et al. (1983) reported the modification of a cysteine residue of ATP synthase by a phospholipid-derived photoaffinity label; very low photoincorporation was observed when the modified protein was isolated under acidic conditions, and all of the label was lost upon exposure to the very acidic conditions used for cleavage by CNBr. Finally, 8-azidoATP has been reported to react reversibly with one or more cysteine residues of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, based upon the ability of dithiothreitol to restore the loss in enzymatic activity (El-Maghrabi et al., 1987). However, this interpretation is complicated by the presence of high concentrations of dithiothreitol during photolysis, conditions which would be expected to reduce the azidoATP to a nonphotoactive derivative (Staros et al., 1978).

We have shown that 8-N_3GTP fulfills all of the criteria for a specific affinity label for PEPCK and that enzyme inactivation by the photoprobe is caused by the formation of an intramolecular disulfide bridge. The sum of the data in this study provides evidence for the existence of a pair of spatially proximal cysteine residues within or near the GTP-binding site of the enzyme; modification of at least one of these cysteines causes a pronounced loss in catalytic activity. Because previous studies have provided evidence for the existence of a vicinal thiol pairing in the native enzyme (Carlson et al., 1978), it is likely that the cysteines that make up the disulfide are initially proximal in the GTP-binding site of the native enzyme and are not juxtaposed following a conformational change induced upon the binding of 8-N_3GTP or upon irradiation. The identification of the half-cystines that compose the disulfide bridge formed by 8-N_3GTP would provide a more detailed picture of the GTP-binding site of PEPCK and also yield information about the folding of the polypeptide chain. There are several characteristics of Cys-288 that would make it a likely candidate as one member of the disulfide pair. Cys-288 is hyperreactive, it is essential for activity, its modification by a maleimide-based sulfhydryl reagent can be nearly completely prevented by GTP, and it likely exists in a vicinal thiol pairing (Carlson et al., 1978; Lewis et al., 1989).

To our knowledge, these data constitute the first report of the specific induction of a cystine disulfide by a photolyzed aryl azide. It is of interest to note that the inactivation of pyruvate kinase by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine occurs through a similar mechanism, in which the formation of an intramolecular cystine disulfide causes displacement of label (Annamalai & Colman, 1981). A number of nucleotide-binding proteins are known to have reactive or critical cysteine residues within or near their active sites. Further characterization of the types of reactions that can occur between a cystine and reactive photogenerated derivatives of aryl azides in general, and of 8-azidopurines in particular, would prove useful in the application of nucleotide-based photoaffinity labels.

Acknowledgments

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[5] The reaction between the thiol and the ring insertion product of the photolyzed aryl azide was proposed to result in attachment through an S–C bond (Bayley & Staros, 1984); however, it is uncertain whether nitrene insertion into the already nitrogen-rich imidazole portion of the purine ring of GTP would result in a reactive derivative that would form an S–C bond or an S–N bond. Because the known chemistry of an S–N bond is consistent with our results, we hypothesize that this bond is formed between PEPCK and photolyzed 8-N_3GTP regardless of whether or not the photolyzed azide rearranges.

[6] F. A. Davis, Drexel University, personal communication.

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