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A Vicinal Dithiol Containing an Essential Cysteine in Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate) from Cytosol of Rat Liver†

Gerald M. Carlson,‡ Giovanna Colombo,§ and Henry A. Lardy*

ABSTRACT: The highly purified form of phosphoenolpyruvate carboxykinase (PEPCK) contained 13 thiols (all in the reduced state) per 72 000 daltons. Modification of the enzyme with equimolar 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) caused rapid formation of a cystine disulfide bridge and an even more rapid loss of enzymatic activity. Formation of the cystine bridge proceeded about 25 times faster than formation of the analogous intramolecular disulfide of dithiothreitol induced by Nbs_2. *o*-Iodosobenzoate, Cd^{2+}, and the 2,3-dimercapto-1-propanol complex of arsenite were potent, time-dependent, irreversible inhibitors of PEPCK. The inactivation by arsenite-2,3-dimercapto-1-propanol and *o*-iodosobenzoate was first order with respect to both time and inhibitor concentration. The sum of these data indicates the existence in PEPCK of a critical cysteine that is in a vicinal dithiol grouping with a second cysteine. PEP protected against cystine bridge formation induced by equimolar Nbs_2 but not against the extent of inactivation. In the presence of PEP, the modification by Nbs_2 of one cysteine/mol of enzyme ($k = 1.2 \times 10^6$ M^{-1} min^{-1} at pH 7.2) caused nearly complete inactivation. Replacing the bulky 5-thio-2-nitrobenzoate moiety with cyanide did not result in any reactivation. This critical, cyanylated cysteine was determined to be 44% of the distance from the amino terminus.

Phosphoenolpyruvate carboxykinase (GTP) (PEPCK, EC 4.1.1.32)[1] from a variety of species and tissues is sensitive to sulfhydryl reagents. *p*-Hydroxymercuribenzoate inhibits PEPCK isolated from the mitochondria of chicken liver (Utter

† From the Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received July 10, 1978.* This study was supported in part by Grants AM10334 and AM20678 from the National Institutes of Health.

‡ Present address: Department of Chemistry, University of South Florida, Tampa, Florida 33620. Recipient of a Postdoctoral Fellowship from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM05234).

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[1] Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase (GTP); Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); Nbs, 5-thio-2-nitrobenzoate; BAL, 2,3-dimercapto-1-propanol; EDTA, ethylenediaminetetraacetate; OAA, oxalacetate; PEP, phosphoenolpyruvate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TEA, triethanolamine; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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et al., 1954), from yeast (Cannata & Stoppani, 1963a,b), the cytosol of oyster (Reeves, 1975), and the mitochondria of pig liver (Chang & Lane, 1966) and sheep kidney (Barns & Keech, 1968). Only with this last enzyme has a detailed analysis of the reactivity and possible role of enzymatic cysteines been performed (Barns & Keech, 1968, 1972).

Even though the chemistry of the cysteinyl residues of PEPCK from the cytosol of rat liver has not been studied, a number of processes affecting that enzyme have been reported to be influenced by exogenous thiols. For instance, thiols have been used to stabilize the purified enzyme (Ballard & Hanson, 1969) and the enzymatic activity in hepatic supernatants of rats treated with tryptophan (Foster et al., 1967). Bentle & Lardy (1976) found that in the presence of Fe^{2+}, thiols were needed for the stimulation of activity by sulfate and the inhibition by orthophosphate. Reduced glutathione protects against inactivation promoted by acetaldehyde (Baxter, 1976), whereas L-cystine enhances inactivation caused by heat, trypsin, or a microsomal fraction (Ballard & Hopgood, 1976). Johnston (1970) reported that increasing the ratio of oxidized to reduced glutathione caused inhibition of Mn^{2+}-stimulated PEPCK activity before the basal activity was suppressed. Thus, many factors that affect the activity of this enzyme act in concert with thiols. An understanding of the mechanisms through which these interactions occur awaits elucidation of the sulfhydryl chemistry of this enzyme.

The purpose of the present study is to provide reference data on the reactive cysteinyl residues of PEPCK isolated from the cytosol of rat liver. Our data indicate the existence of a vicinal dithiol in the enzyme with at least one of the two cysteines being essential for catalytic activity.

Experimental Procedure

If not stated below, reagents were from sources previously listed (Colombo et al., 1978). A 50 mM stock solution of 2,3-dimercapto-1-propanol (BAL) from Aldrich was made up daily in cold H_2O. The concentration of this solution was verified by titration with Nbs_2. The arsenite–BAL conjugate was made several minutes before use by mixing equimolar amounts of the two reagents in H_2O. The concentration of this conjugate was kept under 0.5 mM, because after several minutes at that concentration, or above, the solutions became hazy. *o*-Iodosobenzoate (Sigma) was made up as a 5 mM stock solution in 7.5 mM NaOH and then diluted into H_2O prior to use. A 15 mM stock solution of $K^{14}CN$ (47.8 Ci/mol, New England Nuclear) was prepared in 0.25 M Tes (pH 7.2).

PEPCK was purified as described previously, with chromatography on agarose–hexane–GTP as the final step (Colombo et al., 1978). The elution salt and exogenous thiols were removed by passage over a Sephadex G-25 column—from which the trailing edge of the protein peak was not used. Separation of the protein from exogenous thiols was verified by measuring the reaction with Nbs_2 in fractions immediately following the protein peak. Only enzyme that had a specific activity of at least 14 μmol of PEP carboxylated min^{-1} mg^{-1} at 25 °C was used in this study. PEPCK concentration was determined spectrophotometrically using an absorbancy index of 16.6 at 280 nm for a 1% protein solution (Colombo et al., 1978). Molarity of PEPCK solutions was calculated assuming a molecular weight of 7.2×10^4. The molar ratios of sulfhydryl reagents of PEPCK discussed in this paper will refer, in all cases, to the actual molarity of PEPCK and not to the molarity of its cysteinyl residues.

Enzymatic activity was routinely assayed in the reverse direction (carboxylation of PEP to form OAA) in the presence of IDP (1 mM) and Mn^{2+} (2.25 mM) as described previously (Colombo et al., 1978). In some instances, however, this assay was performed with MgIDP (4 mM Mg^{2+}, 1 mM IDP) plus Mn^{2+} or Co^{2+} at 40 μM. Assays that monitored the formation of PEP or pyruvate were those described by Colombo et al. (1978). When Mn^{2+} or Co^{2+} were included in the assay for PEP formation, they were at a concentration of 40 μM.

Modifications of PEPCK with Nbs_2 and 4,4′-dithiopyridine were carried out at 25 °C and quantitated as detailed earlier (Colombo et al., 1978). Cyanylation with [^{14}C]cyanide was performed on enzyme which was first modified by equimolar Nbs_2 in the presence of PEP (5 mM). After the release of Nbs had plateaued, an aliquot of modified enzyme was incubated for 30 min at pH 7.2 and 20 °C with roughly a 200-fold molar excess $K^{14}CN$ over enzyme. This cyanylated enzyme was dialyzed extensively against 10 mM Tes (pH 7.2), 5% glycerol, 0.1 mM EDTA, 5 mM PEP until radioactivity was no longer found in the dialysis buffer. PEP was then removed from the cyanylated enzyme by passage over a Sephadex G-25 column equilibrated with the dialysis buffer minus PEP. Fractions containing peak radioactivity were pooled, and protein concentration and [^{14}C]cyanide incorporation were determined. Cleavage of the cyanylated protein at the modified cysteine was performed by increasing the pH to 9.5 in the presence of 0.9% $NaDodSO_4$, followed by incubating at 60 °C for 30 min and 22 °C for 18 h (cf. Stark, 1977). $NaDodSO_4$–gel electrophoresis and subsequent determination of molecular weights were described previously (Colombo et al., 1978). The peptide bands, after being cut from the gels, were decolorized and solubilized by heating for 1 h at 100 °C with 0.1 mL of 30% H_2O_2. ^{14}C was counted after the addition of 10 mL of Aquasol scintillation fluid.

Results

Titrations with Nbs_2 and 4,4′-Dithiopyridine. In the preceding paper (Colombo et al., 1978), it was shown that titration of PEPCK from cytosol of rat liver with excess Nbs_2 or 4,4′-dithiopyridine under denaturing conditions led to the modification of 13 cysteine residues. In fact, all the cysteines in the enzyme were present in the reduced state. The availability of a homogenous enzyme of high specific activity allowed us to determine how many of those 13 residues were essential for catalytic activity to be expressed. In order to correlate cysteine modification with enzymatic activity, titrations under nondenaturing conditions were performed with low concentrations of Nbs_2.

When the enzyme was incubated with approximately equimolar Nbs_2 there was a rapid release of not 1, but 2 mol of Nbs and an even more rapid decrease in enzymatic activity. It should be emphasized that, throughout this paper, comparisons of the molarity of sulfhydryl reagents with that of the enzyme will be based on the actual molarity of PEPCK and not on the molarity of its cysteinyl residues. As can be seen in Figure 1, incubation of 0.87 μM PEPCK with 0.89 μM Nbs_2 caused 1.36 mol of Nbs to be released/mol of enzyme in the first 0.6 min of the incubation. The residual activity at this time was only 6% of the original activity. After 5 min the reaction was complete. The activity plateaued at about 3% of the original value, and 2.0 mol of Nbs was released/mol of enzyme. In other experiments with approximately equimolar Nbs_2 the residual activity plateaued as low as 1.8%, and there were always twice as many moles of Nbs released as Nbs_2 added. These findings suggest that the Nbs_2 first attacks a reactive cysteine, causing loss of enzymatic activity and release of 1 mol of Nbs. Following this, a vicinal cysteine must attack the mixed function disulfide between PEPCK and Nbs, forming a cystine disulfide and releasing the second mole of Nbs.

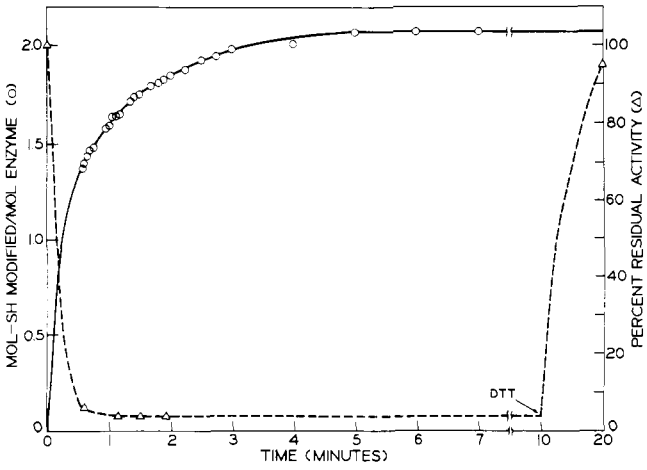

FIGURE 1: Modification and inactivation of PEPCK by approximately equimolar Nbs_2. The reaction included PEPCK (0.87 μM), buffer (0.1 M Tes, pH 7.2), glycerol (2%), and EDTA (0.12 mM) and was initiated by the addition of Nbs_2 (0.89 μM). The modification was followed by measuring the increase in absorbance at 412 nm, from which was continually subtracted the absorbance of a blank containing all the components of the reaction except enzyme. Enzyme activity was measured in the direction of OAA synthesis in the presence of Mn^{2+} with 5-μL aliquots removed at the indicated intervals. The dilution from the incubation to the assay was 200-fold. After 10 min, dithiothreitol (5 mM) was added to the reaction: (○) mol of -SH modified/mol of enzyme; (△) percent residual activity.

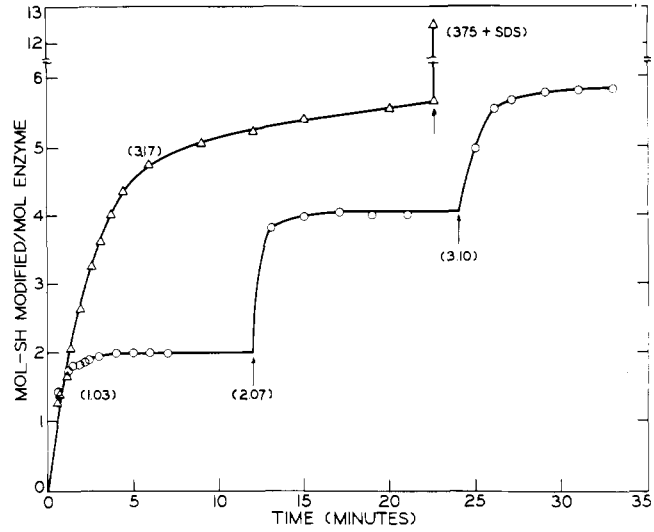

FIGURE 2: Formation of three cystine bridges in PEPCK induced by a threefold molar excess of Nbs_2. The Nbs_2 in this experiment was added in three equimolar increments (○) or as just one addition at a 3.17-fold excess (△). The rate of modification was followed as described in the legend to Figure 1. The reaction carried out with the three stepwise additions of Nbs_2 contained PEPCK (0.95 μM), buffer (0.1 M Tes, pH 7.2), glycerol (1.5%), and EDTA (0.12 mM). At 0, 12, and 24 min, aliquots of Nbs_2 were added so that the molar ratio of Nbs_2 to PEPCK was that indicated by the numbers in parentheses. The modification by the single addition of Nbs_2 at a 3.17-fold molar excess (△) was conducted under similar conditions, except that EDTA was 1 mM. After 22.5 min, the remaining cysteines were titrated by adding sodium dodecyl sulfate (SDS) (0.3%) and an additional 375-fold molar excess of Nbs_2 and following the reaction until it plateaued at 12.5 mol of Nbs released per mole of enzyme.

Assuming that modification by Nbs_2 of the first, and essential, cysteine occurs through a fast second-order reaction, as the rapid loss of activity suggests, then the subsequent formation of the cystine bridge can be treated as an intramolecular first-order process. When the data of Figure 1 were treated in this manner, i.e., log (twice the concentration of Nbs_2 added minus the concentration of Nbs released) vs. time, a straight line was obtained that described a first-order process having an apparent rate of 0.79 min^{-1}. Similar rates were obtained from other titrations at pH 7.2 and 7.6. At the same concentrations and conditions as those of Figure 1, modification by equimolar Nbs_2 of dithiothreitol and formation of the intramolecular disulfide proceeded at approximately $1/25$ the rate of the analogous reaction with PEPCK. Whereas formation of the enzymatic cystine bridge was 75% complete in about 0.75 min, 29 min were required to obtain the same amount of oxidized dithiothreitol. Because the rate-limiting step in the formation of oxidized dithiothreitol is more likely to be the second-order reaction with Nbs_2 rather than the first-order intramolecular disulfide formation, it is probably not valid to directly compare rate constants for the modification of PEPCK and dithiothreitol by equimolar Nbs_2. It is obvious, however, that the reaction with PEPCK proceeds much faster.

The enzyme inactivated by Nbs_2 is easily reactivated by incubation with dithiothreitol. Addition of 5 mM dithiothreitol to the reaction cuvette resulted in greater than 90% reactivation in the experiment described in Figure 1. The time course of reactivation in that figure was taken from separate experiments in which 80–100% reactivation by dithiothreitol required 10 to 15 min.

In order to study sulfhydryl chemistry over a wider range of pH, titrations were also performed with 4,4′-dithiopyridine (Grassetti & Murray, 1967). Analogous to titrations with equimolar Nbs_2, 2 mol of 4-thiopyridone was released for each mole of 4,4′-dithiopyridine employed. Thus, both compounds induced the formation of a cystine bridge in PEPCK. Modification of PEPCK (0.7 μM) with an approximately threefold molar excess of 4,4′-dithiopyridine at pH 6.6 caused a much slower rate of inactivation than was achieved with Nbs_2. The release of 2 mol of 4-thiopyridone/mol of enzyme required more than 20 min. This slow rate allowed the activity to be correlated with the number of sulfhydryls modified. It was found that extrapolation to zero activity correlated with 2 mol of cysteine modified/mol of enzyme; the same result was obtained at pH 6.0 and 7.1. Inasmuch as there was a severalfold excess of 4,4′-dithiopyridine over enzyme, we have no way of knowing if the extrapolation to zero activity at 2 mol of modified cysteine represents a single disulfide bridge, leading to loss of activity, or two discrete modified cysteines. In view of the results obtained with Nbs_2, however, it seems reasonable to expect that the loss of activity is caused by formation of a single cystine bridge. If this interpretation is correct, the rate of formation of the cystine bridge under these conditions must occur more rapidly than the reaction of the essential cysteine with 4,4′-dithiopyridine.

In addition to formation of the first cystine bridge associated with the loss of activity, treatment of the enzyme with additional Nbs_2 resulted in at least two more cystine bridges being formed. When PEPCK was titrated with an approximately threefold molar excess of Nbs_2, nearly 6 mol of Nbs was released/mol of enzyme within 30 min (Figure 2). Additional incubation resulted in exactly twice as many moles of Nbs released as Nbs_2 added (results not shown). Furthermore, it did not matter if the threefold molar excess was added at one time or in three equimolar increments. The numbers in parentheses in Figure 2 refer to the molar excess of Nbs_2 over PEPCK present during the indicated intervals. The same results were obtained with titration by 4,4′-dithiopyridine.

We considered the possibility that the three apparent cystine bridges might be artifacts caused by contamination of the enzyme with nonenzymatic thiols, or other substances, that

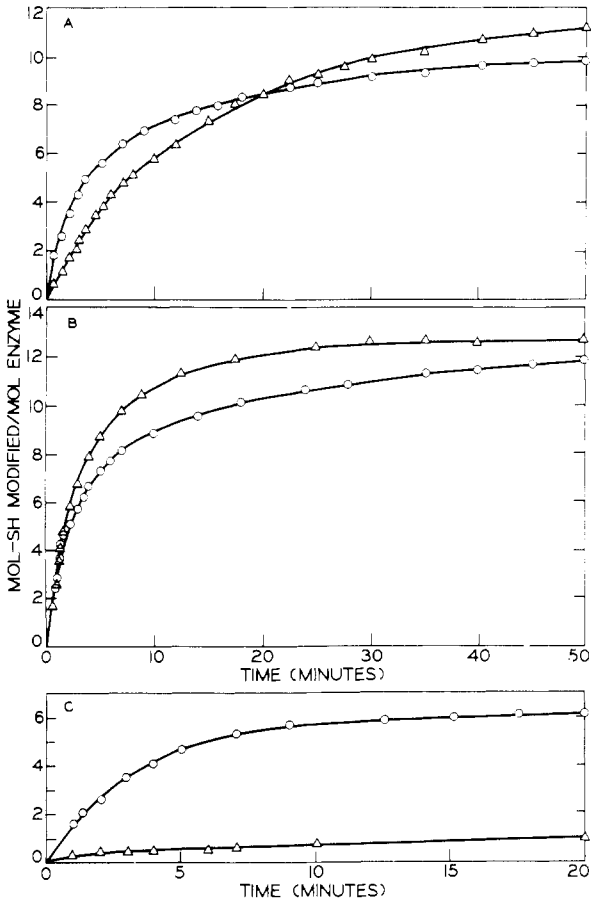

FIGURE 3: Effect of $NaDodSO_4$ on the rate of thiol modification of PEPCK by Nbs_2 at pH 7.1 and 7.6. All reactions included PEPCK (0.37 μM), buffer (0.1 M Tes), and glycerol (1%). Where included, the $NaDodSO_4$ concentration was 0.9%. The concentration of EDTA was 0.1 mM in the absence of $NaDodSO_4$ and 0.8 mM in its presence. The reactions were initiated by the addition of either a 115-fold, or 3.1-fold molar excess of Nbs_2, and the modifications were followed as described in the legend to Figure 1: (A) modification at pH 7.1 with a 115-fold molar excess of Nbs_2; (B) modification at pH 7.6 with a 115-fold molar excess of Nbs_2; (C) modification at pH 7.1 with a 3.1-fold molar excess of Nbs_2. (○) Minus $NaDodSO_4$; (△) plus $NaDodSO_4$.

could attack Nbs_2. However, the addition of $NaDodSO_4$ and a large excess of Nbs_2 after nearly six cysteines/mol of enzyme had been modified resulted in the modification of only 6.5 additional cysteines (Figure 2). Increasing the EDTA concentration to 1.1 mM did not change the results. In no experiment did we measure more than 13 total cysteines. This indicates that three cystine bridges are formed and that the released Nbs is not derived from the breakdown of Nbs_2 by contaminants. Other evidence demonstrating this fact is summarized in the Discussion.

Evidence to support the existence of a class of sulfhydryl groups whose activity is dependent upon enzyme conformation was obtained from titrations with Nbs_2 at pH 7.1 and 7.6 in the presence and absence of $NaDodSO_4$. At pH 7.1 the modification of enzymatic cysteines by a 115-fold molar excess of Nbs_2 initially proceeded more rapidly in the absence than in the presence of 0.9% $NaDodSO_4$ (Figure 3A). After a time, the rate of modification in the absence of $NaDodSO_4$ became slower than in the presence of $NaDodSO_4$, and a crossover occurred at approximately 8 mol of cysteine modified/mol of enzyme. At pH 7.6, however, the rate of modification in the absence of $NaDodSO_4$ was at no time faster than the rate in the presence of $NaDodSO_4$ (Figure 3B). If the amount of Nbs_2 was decreased to only a threefold molar excess, then the modification at pH 7.1 in the presence of $NaDodSO_4$ became very slow compared to the reaction in the absence of $NaDodSO_4$ (Figure 3C). One possible interpretation of these results is that there is a group of enzymatic cysteines which are highly reactive because of low acid dissociation constants caused by their microenvironment in the protein. At low pH, destruction of the enzyme's conformation by $NaDodSO_4$ would raise those pK_a values and lower the cysteinyl reactivity. At a higher pH, more cysteines would already be deprotonated, and destruction of the microenvironment by $NaDodSO_4$ would not result in the net lowering of reactivity of the total cysteine pool.

The rate of modification by Nbs_2 is apparently influenced a great deal by ionic strength. With 100 mM Tes at pH 7.2, the modification by a threefold molar excess of Nbs_2 was 50% complete in about 2 min; when 100 mM KCl was included, it took eight times longer for the reaction to reach 50% completion. However, in both cases, three cystine bridges were formed per mole of enzyme at completion. That this effect was due to ionic strength and not to specific effect of KCl was suggested by the influence of other salts on the sulfhydryl reagents, as described in the following sections.

With either Nbs_2 or 4,4'-dithiopyridine, under a variety of conditions, there did not seem to be any major difference in the inactivation curves when the assays of enzymatic activity were varied. Some of the assays employed were: the formation of pyruvate; the formation of oxalacetate in the presence of Mn^{2+}, $Mg^{2+} + Co^{2+}$, or $Mg^{2+} + Mn^{2+}$; and the formation of phosphoenolpyruvate in the presence of Mg^{2+} or $Mg^{2+} + Co^{2+}$.

Inactivation by Arsenite-BAL. Titration with equimolar Nbs_2 led to inactivation of the enzyme and formation of a cystine disulfide bond. Were the thiols involved in the cystine bridge formation originally near each other, or did formation of the bulky mixed function-disulfide intermediate cause a conformational change which brought the thiols close together? If reagents that are specific for vicinal thiols cause inhibition of the enzyme, it would imply that the thiols involved in the bridge formation were initially proximal.

Inhibition by arsenite has often been used as a criterion for the existence of a vicinal dithiol (Gaber & Fluharty, 1968). The inhibition is evidently due to the formation of a cyclic dithioarsenite complex between arsenite and the vicinal thiols of the enzyme. Arsenite by itself at concentrations up to 5 mM (a 1.6×10^4 fold molar excess over enzyme) had no effect on enzymatic activity when incubated with the enzyme prior to the assay. Similar concentrations of arsenite also did not affect activity when added directly to the assay mixture.

With many enzymes the dithiol, BAL, potentiates the action of arsenite [presumably through an exchange reaction between vicinal enzymatic sulfhydryls and the cyclic dithioarsenite complex formed by arsenite and BAL (Fluharty & Sanadi, 1961)]. Indeed, this combination was effective with PEPCK. Incubating the enzyme with excess BAL and arsenite (arsenite-BAL, equivalent molar amounts of each) resulted in rapid inactivation. The inactivation obtained with a 150- and a 300-fold molar excess of arsenite-BAL over enzyme is shown in Figure 4 as a semilogarithmic plot. At a given arsenite-BAL concentration, the inactivation obeyed pseudo-first-order kinetics with respect to time until at least 98% of the activity was lost. Superimposable decay curves were obtained when activity was assayed in the forward direction with Mg^{2+} or in the reverse direction with Mn^{2+}. Additional incubation resulted in complete inactivation. The combination of arsenite and dithiothreitol had no effect on enzymatic activity, and BAL or dithiothreitol by themselves did not affect activity under these conditions (Figure 4).

The rate of enzyme inactivation (expressed as $1/t_{0.5}$) was

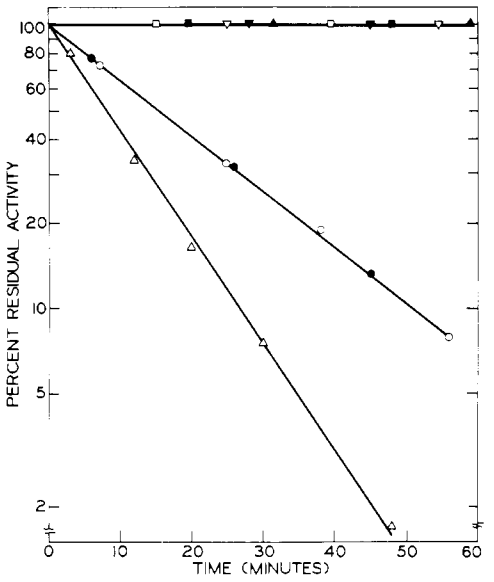

FIGURE 4: Pseudo-first-order inactivation of PEPCK by arsenite-BAL. The standard incubation at 22 °C contained buffer (20 mM TEA, pH 7.3), glycerol (11%) and EDTA (30 μM) and was initiated with PEPCK (0.40 μM). At the indicated intervals, 40-μL aliquots were removed and assayed for activity. The dilution from the incubation to the assay was 25-fold. Unless otherwise noted, assays were performed in the direction of OAA formation in the presence of Mn^{2+}. In addition to the standard incubation mixture, individual tubes contained the following: (■) H_2O; (□) 0.36 mM $NaAsO_2$; (▼) 0.12 mM dithiothreitol; (▲) 0.12 mM dithiothreitol + 0.12 mM $NaAsO_2$; (▽) 0.12 mM BAL; (△) 0.12 mM BAL + 0.12 mM $NaAsO_2$; (○, ●) 0.06 mM BAL + 0.06 mM $NaAsO_2$ assayed in the reverse direction with Mn^{2+} (○), or forward direction with Mg^{2+} (●).

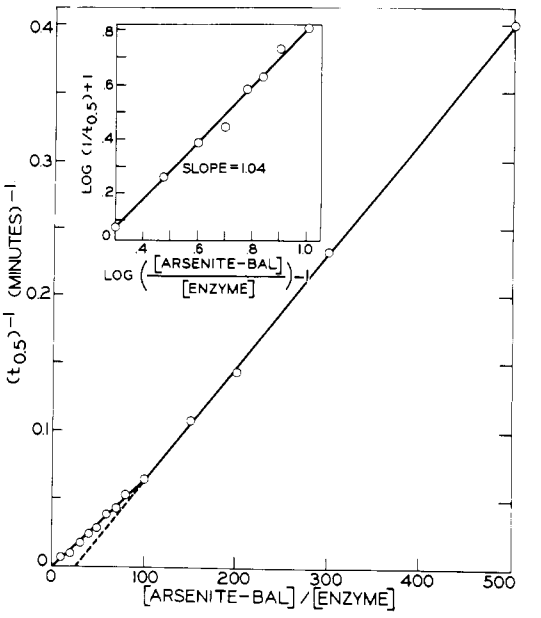

FIGURE 5: Determination of the concentration dependence of PEPCK inactivation by arsenite-BAL and the order of the inactivation process with respect to the concentration of arsenite-BAL. The incubations at 22 °C contained PEPCK (0.35 μM), buffer (11 mM Tes, pH 7.2), glycerol (10%), and EDTA (30 μM) and were initiated with the addition of various concentrations of equimolar arsenite-BAL. The concentration of the arsenite-BAL conjugate ranged from 3.5 (tenfold excess) to 175 μM (500-fold excess). At appropriate intervals 50-μL aliquots were removed from the incubation and assayed for enzymatic activity in the reverse direction with Mn^{2+}. The dilution from the incubation to the assay was 20-fold. Residual activity was plotted against time as in Figure 4, and the $t_{0.5}$ of inactivation was determined. The rate of inactivation ($1/t_{0.5}$) was then plotted in this figure against the arsenite-BAL concentration, expressed as a molar ratio to enzyme. The inset shows the determination of the order of the reaction between PEPCK and arsenite-BAL, with respect to arsenite-BAL, at molar ratios of arsenite-BAL to PEPCK up to 100. The data of Figure 5 are plotted as log of the reciprocal of the half-time of inactivation against log (concentration of arsenite-BAL divided by the concentration of the enzyme). The slope of the plot is 1.04.

proportional to the concentration of arsenite-BAL but in a biphasic manner. At low concentrations of arsenite-BAL, the relative rate of inactivation was slightly slower than that observed at higher concentrations (Figure 5). Arsenite has been shown to form unstable complexes with low-molecular-weight alcohols (Cap, 1861; Wilson & Silman, 1977). Inasmuch as these incubations were performed in 10% glycerol, it seems reasonable to assume that the glycerol acted to decrease the concentration of available arsenite at the lower concentrations of arsenite-BAL employed in the experiments described by Figure 5.

The order of an inactivation reaction with respect to inhibitor concentration can be determined from a plot of log ($1/t_{0.5}$) against the log of the inhibitor concentration (Levy et al., 1963). Such a plot for arsenite-BAL is shown in the inset of Figure 5. The slope of that line is equal to the number of molecules of inhibitor that interact with each active site to produce an inactive enzyme-inhibitor complex. The inset shows the data obtained at the lower concentrations of arsenite-BAL, and the line has a slope of 1.04. When the data for higher concentrations of arsenite-BAL were plotted, a slope of 1.14 was obtained. Thus, one molecule of arsenite-BAL interacted with the active site of PEPCK to cause an inactivation which was first order with time.

The inactivation caused by arsenite-BAL was greatly influenced by ionic strength. When NaCl or $NaNO_3$ was included in the incubation mixture described in the legend to Figure 4, there was a decrease in the rate of inactivation ($1/t_{0.5}$). Addition of 50 mM NaCl caused the rate of inactivation to decrease to 53% of that observed in its absence. Inclusion of 100 mM NaCl or $NaNO_3$ decreased the rate an additional 13%. From 100 to 500 mM NaCl, however, there was little additional change in the rate of inactivation.

The arsenite-BAL can be considered to be a kinetically irreversible inhibitor. At the lowest concentration of arsenite-BAL tested (3.5 μM), a 33-fold dilution into the assay still resulted in linear activity assays over a long period of time; i.e., dilution of the inhibitor did not cause reactivation. The inactive enzyme could be partially reactivated with either dithiothreitol or BAL. After complete inactivation with 150-fold excess arsenite-BAL, 20 mM dithiothreitol was able to restore 33% of the original activity in 2 h (results not shown). In a 25-min period, 18 mM BAL restored 12% of the activity. Complete reversal of the arsenite-BAL inactivation was never obtained.

Inhibition by Cd^{2+}. The data from titrations with Nbs_2 and inactivation with arsenite-BAL indicate the presence of a vicinal dithiol in PEPCK containing at least one essential cysteine. Another criterion often used to infer the presence of a vicinal dithiol is inhibition by low concentrations of Cd^{2+} (Gaber & Fluharty, 1968). Snoke et al. (1971) reported that the activity of PEPCK in hepatic supernatants was affected by inclusion of Cd^{2+} in the reaction mixture for assaying in the forward direction with Mg^{2+}. They found that 0.1 and 1 mM Cd^{2+} stimulated activity, whereas 10 mM Cd^{2+} caused inhibition. Using purified enzyme and treating with Cd^{2+} prior to the assay, we have studied the effect on activity of much lower concentrations of Cd^{2+}.

When purified enzyme (0.35 μM) was incubated for various times at 22 °C with 30 μM EDTA and increasing concentrations of Cd^{2+} from 40 to 100 μM, there was a progressive in-

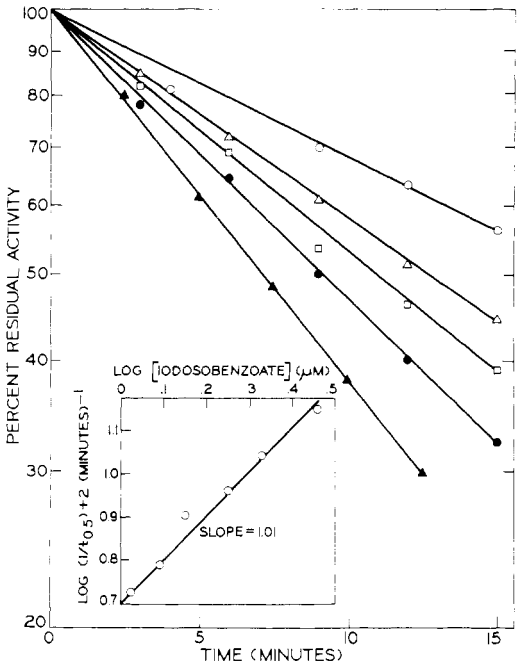

FIGURE 6: Kinetics of the inactivation of PEPCK by *o*-iodosobenzoate. The incubations at 22 °C contained PEPCK (0.35 μM), buffer (11 mM Tes, pH 7.2), glycerol (10%), and EDTA (30 μM) and were initiated with various concentrations of *o*-iodosobenzoate from 1.05 (threefold excess) to 2.8 μM (eightfold excess). The five inactivation plots, from top to bottom, correspond to a three-, four-, five-, six-, and eightfold molar excess of *o*-iodosobenzoate over PEPCK. At the indicated times 50-μL aliquots were removed and assayed for activity in the reverse direction with Mn^{2+}. The dilution from the incubation to the assay was 20-fold. The inset shows the determination of the order of the reaction between PEPCK and *o*-iodosobenzoate with respect to *o*-iodosobenzoate. The data of Figure 6, plus an additional inactivation with a 3.5-fold molar excess of *o*-iodosobenzoate, are plotted as log of the reciprocal of the half-time of inactivation in minutes against log of the μM concentration of *o*-iodosobenzoate. The slope of the plot is 1.01.

hibition of activity as measured in the forward direction in the presence of Mg^{2+}. The effect of Cd^{2+} on enzymatic activity was time dependent, and throughout this range of Cd^{2+} concentrations a given concentration was first stimulatory and then inhibitory. The time necessary for inhibition to become apparent depended upon the concentration of Cd^{2+}. Inhibition occurred in less than 5 min with 80 μM Cd^{2+}/30 μM EDTA, but with 40 μM Cd^{2+}/30 μM EDTA inhibition was not apparent until approximately 20 min of incubation. Incubation for 7 min prior to assay with 30 μM EDTA and 50, 60, 70, 80, and 100 μM Cd^{2+} caused, respectively, 37, 58, 76, 82, and 90% inhibition of the basal activity.

The inhibition by Cd^{2+} was only partially reversible by EDTA (3 mM) or dithiothreitol (45 mM). When enzyme with 1% residual activity was incubated up to 18 h with either EDTA or dithiothreitol, there was a reactivation to 20% of the basal activity. Even though the action of Cd^{2+} on PEPCK is somewhat complex, it is obvious that very low concentrations of this metal dramatically inhibit enzymatic activity. This finding is consistent with the presence of a critical vicinal dithiol.

Inactivation by o-Iodosobenzoate. In order to learn more about the reactivity of the critical cysteine(s), the specificity of its modification, and its involvement in the vicinal dithiol grouping, a different type of sulfhydryl reagent was used as an inactivating agent. *o*-Iodosobenzoate, a commonly used oxidant for sulfhydryl groups, is usually presumed to cause formation of a cystine disulfide bond (Webb, 1966). Preliminary studies with fixed concentrations of *o*-iodosobenzoate and single times of inactivation have shown that this reagent does inhibit, at least partially, PEPCK from other sources (Cannata & Stoppani, 1963b; Barns & Keech, 1968). We have examined in greater detail the time and concentration dependencies, kinetic order, and reversibility of inactivation by *o*-iodosobenzoate.

Incubation of the enzyme with *o*-iodosobenzoate prior to assay resulted in its time-dependent inactivation. At all concentrations of *o*-iodosobenzoate in Figure 6 the rate of inactivation was first order with respect to time. In separate experiments with higher concentrations of *o*-iodosobenzoate, it was noted that at greater than 70% inactivation the order of inactivation with time exceeded one. The decay curves with a threefold excess of *o*-iodosobenzoate were identical when activity was assayed in the reverse direction with Mn^{2+} or in the forward direction with Mg^{2+}. The rate of inactivation ($1/t_{0.5}$) was directly proportional to the concentration of *o*-iodosobenzoate, and plots of the rate of inactivation against *o*-iodosobenzoate concentration intercept the origin (results not shown). When the log of the rate of inactivation was plotted against the log of the inhibitor concentration, a straight line with a slope of 1.01 was obtained (inset of Figure 6). As was the case with the arsenite–BAL, this slope indicates that one molecule of *o*-iodosobenzoate interacts with each active site of PEPCK to cause inactivation of the enzyme.

The inactivation caused by *o*-iodosobenzoate was influenced by ionic strength in a biphasic manner. When 50 mM NaCl was included in the incubation mixture described in the legend to Figure 6, the rate of inactivation was about doubled. Additional NaCl caused the rate of inactivation to decrease slowly until it reached the control value at approximately 0.25 M salt and 60% of the control value at 0.5 M salt. Whereas inclusion of 50 mM NaCl about doubled the rate of inactivation by *o*-iodosobenzoate, it halved that caused by arsenite–BAL. There was no apparent difference between the effects of $NaNO_3$ and NaCl with either sulfhydryl reagent.

When PEPCK was inactivated with a fivefold excess of *o*-iodosobenzoate to a residual activity of 2%, treatment with 70 mM dithiothreitol for 30 min reversed the loss of activity to 40% of the initial value. In no experiment did dithiothreitol fully reverse the inactivation by *o*-iodosobenzoate. The sum of the data from titrations with Nbs_2 and inhibition by Cd^{2+}, arsenite–BAL, and *o*-iodosobenzoate provides strong evidence for the existence of a vicinal dithiol in PEPCK with at least one of the cysteines being essential for catalytic activity.

Substrate Protection Against Sulfhydryl Reagents. If the essential cysteine is located within the active site, then substrates of PEPCK should depress the rate of reaction between the enzyme and the sulfhydryl reagents. This was, indeed, the case with arsenite–BAL, *o*-iodobenzoate, and Nbs_2. We were unable to test Cd^{2+} because all the substrates have the ability to chelate divalent metal ions.

With a 300-fold molar excess of arsenite–BAL, PEP provided the least protection against inactivation (Table I). The combination of PEP plus IDP gave the greatest protection, and IDP and ITP provided intermediate protection. With all substrates tested, the inactivation by arsenite–BAL was, once again, first order with respect to time for at least 90% inactivation.

With a tenfold molar excess of *o*-iodosobenzoate, PEP again offered less protection against inactivation than the other substrates tested (Table I). The relative protection by PEP was greater against *o*-iodosobenzoate, however, than it was against arsenite–BAL. The inactivation by *o*-iodosobenzoate in the presence of PEP was also first order with time for at least 75%

TABLE I: Protection by Substrates against Effects of Sulfhydryl Reagents on Phosphoenolpyruvate Carboxykinase at pH 7.2.

	$t_{0.5}$ of inact. (min)		equimolar Nbs_2[c]			
substrate[a]	arsenite–BAL[b]	*o*-iodosobenzoate[b]	time req to mod. 0.5 –SH (min)	mol of –SH modif/ mol of enz	% res act. after 1.0 –SH modif	% res act. after plateau reached[i]
none	19.7	16.1	~0.2[d]	2.0	~10[e]	~3
PEP	21.5	35.8	1.2	~1.1[f]	~10	~10[g]
IDP	28.0	>120	9.0	1.9[h]	55	45[h]
ITP	30.4	>120	13.0	1.6[h]	57	53[h]
PEP + IDP	40.2	>120	10.0	1.0[h]	50	49[h]

[a] Substrates were present at 4 mM each in the titrations with Nbs_2, and 5 mM each in the other incubations. [b] Incubations at 22 °C contained, in addition to substrates, PEPCK (0.35 μM), NaCl (0.3 M), EDTA (1.5 mM), Tes (25 mM, pH 7.2), and glycerol (10%) and were initiated with either arsenite–BAL (105 μM, 300-fold molar excess) or *o*-iodosobenzoate (3.5 μM, tenfold molar excess). Assays of enzymatic activity and determinations of $t_{0.5}$ of inactivation were performed as described in the legend to Figure 6. [c] Modifications with Nbs_2 were performed under the conditions described in the legend to Figure 1, except substrates were present where indicated. [d] Occurs too rapidly to measure accurately. [e] Can only be estimated because first sulfhydryl is modified so rapidly. [f] Under various conditions and with different enzyme preparations, this value ranged from 1.0 to 1.3. Values from 1.0 to 1.1 were generally observed under these conditions. [g] This residual activity corresponds to modification of 1.1 mol of cysteine/mol of enzyme. When modification was higher, so also was residual activity (see text). [h] Titrations in the presence of nucleotides proceeded very slowly, and the reactions were arbitrarily stopped after 80 min without certainty that a plateau had been reached. [i] Or after 80 min.

inactivation. With IDP, ITP, and PEP plus IDP, the protection against inactivation by a tenfold molar excess of *o*-iodosobenzoate was so high that inhibition was difficult to measure. The half-time of inactivation was certainly greater than 120 min in all three cases. This high protection is extraordinary considering that *o*-iodosobenzoate is acting as an irreversible inhibitor.

We observed a wide variety of effects when the substrates were included in the titrations of PEPCK with equimolar Nbs_2. PEP decreased the initial rate of cysteine modification and blocked formation of the cystine bridge containing the essential thiol (Table I); i.e., approximately 1 mol of Nbs/mol of enzyme was released in the presence of PEP, as opposed to 2 mol of Nbs released in its absence. The rate of inactivation by Nbs_2 was also decreased in the presence of PEP, but the extent of inactivation was not much different than in its absence. Thus, even though the cystine bridge containing the critical thiol was not formed, an essential thiol was still modified in the presence of PEP. We presume this modified thiol is the same one normally found in the first cystine bridge induced by equimolar Nbs_2. In the absence of primary sequence data, this supposition is difficult to prove. It should be remembered, however, that PEP offered the least protection against inactivation by the other sulfhydryl reagents. There is a further description of the titration in the presence of PEP in the next section. OAA did not protect against formation of the cystine bridge at low ratios of Nbs_2 to enzyme, but it did cause the rate of its formation to be slightly decreased.

The nucleotides IDP and ITP offered even greater protection against the initial modification of enzymatic thiols by equimolar Nbs_2 (Table I). With both nucleotides, however, there was eventually a significant amount of cystine bridge formed, but, inasmuch as the residual activity was so high, the bridge(s) formed had to be other than, or in addition to, the first cystine bridge containing the critical cysteine. Certainly the data in Figure 2 indicate that there are ample opportunities to form other cystine bridges. The combination of IDP plus PEP blocked formation of the cystine bridge (as was seen with PEP alone) but allowed retention of approximately 50% activity (as was seen with IDP alone) (Table I).

MgIDP and MgITP were found to behave similarly to IDP and ITP in the titrations with Nbs_2 (results not shown). This is in contrast to the effects of MnIDP and MnITP. MnITP offered the most effective protection against inactivation by Nbs_2 of all the substrates tested. In one titration of PEPCK with equimolar Nbs_2 at pH 7.6 in the presence of MnITP (0.75 mM Mn^{2+}/0.64 mM ITP), 0.8 cysteine/mol of enzyme was modified in the first 45 min with no loss of activity. In a similar experiment with a 1.7-fold molar excess of Nbs_2 and 4 mM Mn^{2+} and ITP, 1.1 cysteines/mol of enzyme were modified after 24 h with no loss of activity. At pH 6.0 with a 51-fold excess of 4,4′-dithiopyridine in the presence of MnITP (4.5–4 mM), one cysteine was modified after 3.7 h with no loss of activity. MnIDP behaved similarly to MnITP but was not as effective. Mn^{2+} alone at 4 mM caused only a slight decrease in the rate of bridge formation brought about by low concentrations of Nbs_2.

Some titrations with high molar excesses of Nbs_2 were also performed in the presence of PEP (4 mM) at pH 7. With a 127-fold molar excess of Nbs_2, the rate of modification of the first thiol per mole of enzyme was too fast to measure in both the presence and absence of PEP. The enzymatic activity in both cases was nearly abolished after the modification of 1 mol of cysteine/mol of enzyme. Modification of the remaining thiols, however, was dramatically inhibited by PEP. During the first 60 min of the titration only 1 additional mol of cysteine was modified/mol of enzyme in the presence of PEP. In its absence, however, an additional 9.3 mol of cysteine/mol of enzyme was modified. Inclusion of Mg^{2+} or Mn^{2+} with the PEP did not seem to influence the reaction any differently than PEP alone.

Identification of a Single Essential Cysteine. As was noted in the previous section, PEP did not protect against the extent of inactivation caused by Nbs_2, but it did protect against bridge formation induced by a slight molar excess of Nbs_2. We decided to study this phenomenon in detail. When PEPCK was titrated with approximately equimolar Nbs_2 in the presence of PEP under the conditions described in the legend to Figure 7, there was inactivation of the enzyme and release of approximately 1 mol of Nbs. A second-order plot of the reciprocal concentration of remaining Nbs_2 vs. time was linear and yielded a rate constant of 1.2×10^6 M^{-1} min^{-1}. This modifi-

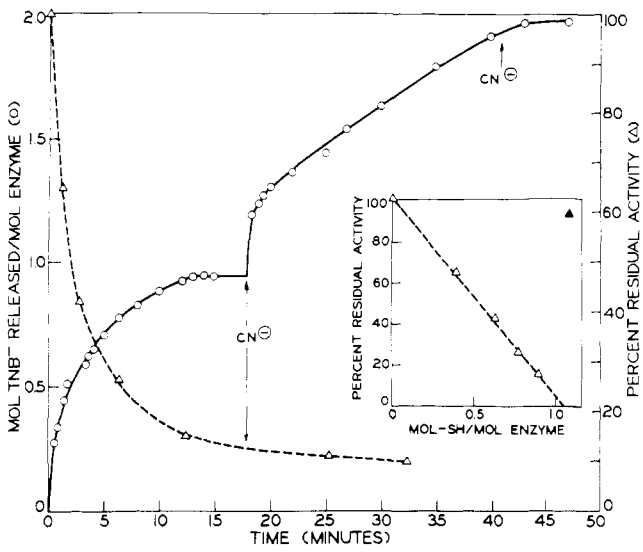

FIGURE 7: Modification and inactivation of PEPCK by equimolar Nbs_2 in the presence of PEP and the subsequent cyanylation of the enzyme. The initial incubation included PEPCK (0.87 μM), buffer (0.1 M Tes, pH 7.0), glycerol (6%), EDTA (0.16 mM), and PEP (3.9 mM) and was begun by the addition of Nbs_2 (0.88 μM). The rate of modification was followed as described in the legend to Figure 1. At the indicated intervals 5-μL aliquots were removed for the assay of enzymatic activity in the reverse direction with Mn^{2+}. The dilution from the incubation to the assay was 200-fold. KCN (1.36 mM) was added to the incubation after 18 min. Release of Nbs and enzymatic activity were monitored as described above. After 41 min, the KCN concentration was increased to 2.7 mM. The inset is a replot of the inactivation observed during the reaction with Nbs_2. The percent residual activity is plotted against the moles of -SH modified per mole of enzyme. The intercept at zero percent residual activity corresponds to 1.04 mol of -SH/mol of enzyme. After 16 min of incubation, an aliquot was removed and further incubated with 80 mM dithiothreitol. The closed triangle (▲) in the inset represents the activity of that aliquot: (○) mol of Nbs released/mol of enzyme; (△, ▲) percent residual activity.

cation occurred about 13 times faster than the analogous reaction with reduced glutathione at the same concentrations and conditions (second-order rate constant of 9.3×10^4 M^{-1} min^{-1}). A plot of residual activity against moles of cysteine modified per mole of enzyme extrapolated to zero activity at about 1 mol of cysteine/mol of enzyme (inset of Figure 7). It was possible to make such a plot because the rate of inactivation by equimolar Nbs_2 was much slower in the presence of PEP (Figure 7) than in its absence (Figure 1). The results of this experiment are in agreement with those outlined in Figure 1, because only 6% residual activity remained after 1.36 mol of cysteine/mol of enzyme had been modified in that experiment. The closed triangle in the inset to Figure 7 shows the nearly complete reactivation caused by dithiothreitol. On the basis of these experiments, we conclude that there is in PEPCK a single cysteine residue whose modification by Nbs_2 results in the inactivation of the enzyme. It is difficult to tell within experimental error if the inactivation is complete, or only nearly complete, when that cysteine is fully modified. This is, of course, not a small point because it would help distinguish a catalytically important residue from one that merely influences the geometry of the active site or affects the binding of substrates. In several experiments the residual activity was less than 3%, but only once did we observe 0% residual activity.

Because the Nbs moiety is so large, we wished to see if modification of the essential cysteine with a much smaller group would allow retention of more enzymatic activity. Cyanide, the smallest uncharged sulfhydryl-blocking group, will attack the mixed-function disulfide between Nbs and cysteine, releasing 1 mol of Nbs for each mole of the *S*-cyano derivative formed (Vanaman & Stark, 1970). After the reaction of PEPCK with equimolar Nbs_2 in the presence of PEP had plateaued, the addition of 1.36 mM KCN caused release of approximately 1 mol of Nbs (Figure 7). Accompanying this exchange, there was, however, absolutely no gain in the activity that was lost during the Nbs_2 titration. Excess KCN did not inhibit the catalytic reaction of untreated enzyme. Titration of the cyanylated enzyme with excess Nbs_2 in the presence of $NaDodSO_4$ caused an additional 12.04 cysteines to be modified.

In order to be absolutely certain that the Nbs released after the addition of cyanide was due to the formation of an *S*-cyano derivative and not a cystine disulfide, we compared the amount of [^{14}C]cyanide incorporated with the amount of Nbs released. In all cases, the moles of Nbs released by either unlabeled or [^{14}C]cyanide were the same as the moles of [^{14}C]cyanide found incorporated into the protein after extensive dialysis, followed by desalting on Sephadex G-25.

Titrations with approximately equimolar Nbs_2 in the presence of PEP were done a total of 17 times under a variety of conditions. Usually the residual activity was lower than that in Figure 7, although occasionally it was higher. We found the higher residual activity in experiments in which a small amount of a cystine bridge was formed that did not influence enzymatic activity (similar to the situation encountered during titrations in the presence of nucleotides). For instance, titration with equimolar Nbs_2 in the presence of PEP plateaued in one case with the release of 1.3 mol of Nbs/mol of enzyme and a residual activity of 24%. Our interpretation of this is that at least two types of modifications had occurred simultaneously: (1) 0.76 mol of the essential cysteine had been modified per mole of enzyme, leaving a residual activity of 24% and releasing 0.76 mol of Nbs, and (2) about 0.24 mol of a nonessential cystine disulfide had been formed releasing the additional 0.5 mol of Nbs. This interpretation is consistent with the finding that the addition of cyanide in that experiment caused release of 0.7 mol of Nbs which coincided with the incorporation of 0.68 mol of [^{14}C]cyanide/mol of protein. Thus, regardless of the residual plateau activity, the inactivation resulted from a modification of a single cysteine.

Cleavage of the [^{14}C]cyanylated enzyme at the modified cysteine was performed as described under Experimental Procedure. $NaDodSO_4$-gel electrophoresis showed the presence, after cleavage, of three protein bands: one with a molecular weight of approximately 70 500 corresponding to the native protein, a second with a molecular weight of about 39 000, and a third band which had a molecular weight of roughly 31 000. Summation of the weights of the two lighter bands equals the weight of the native protein in that run. Determination of radioactivity showed that greater than 80% of the total radioactivity was in the peptide with a molecular weight of 39 000. Only 2% of the radioactivity was found in the peptide with the lower molecular weight. These results indicate that the essential cysteine residue modified by Nbs_2 is located at about 44% of the distance from the amino terminus (cf. Stark, 1977).

Additional evidence supporting the existence of one very reactive cysteine was obtained from titrations with Nbs_2 and 4,4′-dithiopyridine at various pHs. When PEPCK was titrated with a 220-fold molar excess of Nbs_2 at pH 6.1, 6.7, 7.2, and 7.8 (0.1 M Tes-0.1 M Pipes), there was a progressive increase in the rate of sulfhydryl modification as the pH was raised. At every pH, however, there was a single sulfhydryl that was modified at a rate too fast to measure. Plots of $(1/A - B) \ln (B/A)(A - X/B - X)$ vs. time for titrations at all four pHs intercepted the ordinate axis (time zero) at a point corre-

sponding to 1.1 mol of cysteine/mol of enzyme.[2] Titrations with a 185-fold molar excess of 4,4′-dithiopyridine at pH 6.3 and 8.2 (0.1 M TEA–0.1 M KP_i) gave similar results. The intercepts in this case corresponded to 0.96 and 1.2 mol of cysteine/mol of enzyme, respectively.

Discussion

From correlations of residual activity with the extent of modification during titrations of PEPCK with equimolar Nbs_2, in the presence or absence of PEP, it is clear that there is one critical cysteine which must not be modified by Nbs_2 if the enzyme is to retain catalytic activity. Furthermore, this critical cysteine must be vicinal to a second cysteine because equimolar Nbs_2 also caused formation of a cystine bridge. Arsenite–BAL, Cd^{2+}, and *o*-iodosobenzoate, reagents which are commonly presumed diagnostic for a vicinal dithiol, were potent inhibitors of enzymatic activity, thus supporting the idea that the essential cysteine is involved in a vicinal dithiol grouping. The fact that these three diverse inhibitors were effective suggests that the two cysteines in question were initially vicinal, rather than being brought together by a conformational change induced by a modification of the critical cysteine with Nbs_2. We presume that the essential cysteine, defined by modification with Nbs_2, is the same one modified by *o*-iodosobenzoate, arsenite–BAL, and Cd^{2+}. In support of this, the inactivation by *o*-iodosobenzoate and arsenite–BAL was found to be due to binding of a single molecule of those reagents to each active site. In addition, the order of effectiveness of substrates in protecting against inactivation by these two reagents was the same as that observed with equimolar Nbs_2.

When the enzyme was modified with excess Nbs_2 at various pHs between 6.1 and 7.8, there was a single sulfhydryl (the essential cysteine) that was modified at a rate too fast to measure. The rate of formation of the cystine disulfide caused by modification with equimolar Nbs_2 at pH 7.2 was estimated to be 0.79 min^{-1}. The modification of the essential cysteine, which apparently occurred first, must take place at an even faster rate, but in a second-order reaction. The overall reactions to form the cystine bridge occurred about 25-times faster than the analogous reactions with dithiothreitol. Even though PEP slowed the modification of an essential cysteine, the second-order modification by Nbs_2 of that residue occurred about 13-times faster in the presence of PEP than the analogous reaction with reduced glutathione. Not only did we find these high rates of modification surprising, we were equally surprised that, with 13 cysteines in the enzyme, equimolar Nbs_2 exhibited such a high specificity toward the essential cysteine. There are several possible explanations for this apparent specificity, and the real answer could easily be a combination of them. It is possible that most of the sulfhydryls in the native protein are not accessible to the Nbs_2 prior to formation of the first cystine bridge. In support of this is the remarkably high protection by MnITP against Nbs_2. Another possibility is that the high specificity by equimolar Nbs_2 toward the essential cysteine is merely a manifestation of the very high reactivity of that cysteine. Finally, one could assume the existence of a binding site for Nbs_2 near the catalytic site. In this regard, one might think of Nbs as an analogue of 3-mercaptopicolinate, an inhibitor of PEPCK (Kostos et al., 1975; Jomain-Baum et al., 1976) or of OAA. Such a binding site could be further assumed to have a certain amount of hydrophobic character. If this were the case, it would explain why arsenite–BAL was an effective inhibitor, whereas arsenite was not (Fluharty & Sanadi, 1961).

Designating a particular cysteine as "essential" or "critical" implies absolutely nothing about the possible mechanism of the reaction in question. Certainly one of the most difficult problems in protein modification is the proof that an "essential" residue is such because it is a "catalytic" residue. Our definition of a "catalytic" residue is a residue that is located within the active site and directly contributes to the transformation of reactants into products (note that this definition does not attempt to distinguish between binding and the making and breaking of bonds). Is the "essential" cysteine of PEPCK from the cytosol of rat liver a "catalytic" residue? There are numerous pieces of evidence that are consistent with such a role. (1) All of the substrates tested offered protection against the rate of inactivation brought about by the various sulfhydryl reagents. (2) Replacing the bulky Nbs moiety with cyanide caused absolutely no change in enzymatic activity. (3) The inactivation by these sulfhydryl reagents was not due to destruction of the site specific for divalent cations other than Mg^{2+} (Colombo & Carlson, 1978), because the inactivation curves were the same whether assays were in the forward or reverse directions and with various metals present. (4) The residual activity following treatment with the sulfhydryl reagents was either very low or nonexistent. (5) The essential cysteine was found to be highly reactive.

The only other detailed sulfhydryl analysis on PEPCK, of which we are aware, was performed on the mitochondrial enzyme from sheep kidney by Barns and Keech (1968, 1972). Those workers found that *N*-ethylmaleimide acted as an irreversible inhibitor and that the modification of the enzyme was first order with respect to time and inhibitor concentration. Thus, they concluded that there was one essential sulfhydryl group in the enzyme. Of the substrates, IDP offered the most protection against *N*-ethylmaleimide, whereas PEP gave very little protection. Our results obtained with the various sulfhydryl reagents also show the same relative protection by PEP and IDP. From kinetic studies they concluded that the sulfhydryl modified by *N*-ethylmaleimide was associated with the catalytic process as well as substrate binding. Dinitrofluorobenzene was also found to be an irreversible inhibitor, but, because Mn^{2+} protected against dinitrofluorobenzene and not against *N*-ethylmaleimide and because the inactivation was greater than first order with respect to dinitrofluorobenzene concentration, they concluded that dinitrofluorobenzene modified a second essential thiol in addition to the one modified by *N*-ethylmaleimide. We also observed very little protection by Mn^{2+} against modification by Nbs_2. Despite the fact that the mitochondrial and cytoplasmic forms of PEPCK have been shown to be nonidentical proteins with at least some dissimilar properties (Holten and Nordlie, 1965; Ballard and Hanson, 1969; Ballard, 1970), it seems likely that the essential cysteine which we have described is that modified by *N*-ethylmaleimide in the mitochondrial enzyme.

When the enzyme was modified with a threefold molar excess of Nbs_2, 6 mol of Nbs was rapidly released/mol of enzyme. We interpret this as evidence of the formation of three cystine bridges per enzyme molecule. However, inasmuch as the PEPCK is exposed to exogenous thiols during purification and storage, it is critical to prove that the extra 3 mol of Nbs is released by cystine bridge formation and not by reaction of Nbs_2 with contaminants present in the enzyme solutions. We believe there is ample evidence indicating our interpretation is correct. (1) The total number of cysteines titrated by Nbs_2 did not, in any instance, exceed 13/mol of enzyme whether the

[2] Where *A* equals the initial concentration of thiols, i.e., 13 (concentration of PEPCK); *B* equals the initial concentration of Nbs_2; and *X* equals the concentration of product at time *t*.

titrations in the presence of $NaDodSO_4$ were performed before or after bridge formation. (2) The number of cysteines determined by cysteic acid analysis following performic acid oxidation was also 13/mol of enzyme (Colombo et al., 1978). This argues that contaminants are not attacking the Nbs_2. (3) After formation of the first apparent cystine bridge by equimolar Nbs_2, there was virtually no enzymatic activity left. (4) Nbs_2 reacted more rapidly with PEPCK than with dithiothreitol. (5) PEP protected against bridge formation by equimolar Nbs_2 but not against the extent of inactivation. (6) Inactivation by *o*-iodosobenzoate, Cd^{2+}, and arsenite–BAL suggests that at least one vicinal dithiol grouping is present in the enzyme. (7) The rate of modification by a threefold excess of Nbs_2 at pH 7.1 was much faster in the absence of $NaDodSO_4$ than in its presence. If contaminants contributed significantly to the rate, then one would expect $NaDodSO_4$ to have the opposite effect. Consequently, PEPCK can be added to the growing list of proteins in which Nbs_2 induces formation of a cystine disulfide bond. Among other proteins in which this has been observed are brain hexokinase (Redkar and Kenkare, 1975), S-100 protein (Calissano et al., 1976), glyceraldehyde-3-phosphate dehydrogenase from lobster (Wassarman and Major, 1969), and pyruvate kinase from rabbit muscle (Flashner et al., 1972). The unique aspect of the Nbs_2-induced formation of the first cystine disulfide in PEPCK is not the fact that it occurs, but the rapidity with which it occurs.

One might ask why the first cystine bridge containing the essential and most reactive cysteine does not readily form in the absence of Nbs_2. We have stored solutions of the purified enzyme in degassed buffer with EDTA, but no exogenous thiols, for up to 20 days without any change in the activity or cysteine content (Colombo et al., 1978). Perhaps in the absence of Nbs_2 the essential cysteine is complexed with another residue so as to prohibit its interaction with the vicinal cysteine.

The enzyme employed in this study had been freed of contaminating proteins and its less active (inactive?) counterpart by passage over an agarose–hexane–GTP column (Colombo et al., 1978). We wish to emphasize the value of separating active and inactive species of the protein under question in order to correlate modification with activity. Obviously, a protein which is homogeneous by physical criteria will not be fully useful for many biochemical studies if it contains species with different specific activities.

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