POSSIBLE ROLE OF ESSENTIAL THIOL GROUPS*

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The inactivation of beef liver porphobilinogen synthase by the alkylating agents iodoacetate and iodoacetamide has been investigated. The synthase can be inactivated by these reagents only if the enzyme has been activated initially by sulfhydryl reducing agents. Inactivation by both iodoacetate and iodoacetamide was the result of modification of cysteinyl residues. A plot of the incorporation of "C-labeled alkylating reagents versus activity extrapolated to the modification of approximately one fast reacting cysteinyl residue per monomer (M, = 35,000) as responsible for the loss of activity by either iodoacetate or iodoacetamide; the pH of half-maximum alkylation, with both reagents, was about 5.2 indicating that each reagent alkylates a cysteinyl residue of similar high reactivity.

The substrate analogue, levulinic acid, a competitive inhibitor, partially protected the enzyme from inactivation by iodoacetate, suggesting that this alkylation was active site directed. In contrast, levulinic acid potentiates the inactivation caused by iodoacetamide. The product of the reaction, porphobilinogen, however, partially protected the enzyme from inactivation by both iodoacetate and iodoacetamide.

Equilibrium dialysis studies of ["C]levulinic acid binding to the enzyme have shown that native enzyme (thiol-activated or air-oxidized) and the alkylated enzyme derivatives bind the same maximum number of moles of levulinic acid/mol of enzyme (2/285,000 daltons). However, the binding affinities of the native thiol-activated enzyme and the iodoacetamide-inactivated enzyme were 3- to 5-fold greater than those of the air-oxidized and iodoacetamide-inactivated samples of enzyme.

Peptide fingerprints, molecular sieve, and cation exchange chromatography of tryptic digests of the enzyme inactivated by iodol"Cicacetate or by iodol"Cicetamide demonstrated that each reagent alkylated a cysteinyl residue which gave rise to a different radioactive tryptic peptide. Preliminary, this suggests that each reagent alkylated a separate essential cysteinyl residue in the primary sequence. A mechanism is proposed in which cysteinyl residues may participate in the acid/base catalysis required for the enzymic protonation/deprotonation sequences in the synthesis of porphobilinogen.

Porphobilinogen synthase (5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing; EC 4.2.1.24)) has a molecular weight of 285,000 and is composed of eight subunits of mass of 35,000 daltons (1). The enzyme catalyzes the synthesis of PBG from two molecules of δ-aminolevulinic acid (Scheme 1) (for reviews see Refs. 2 to 4). In the proposed mechanism for PBG synthase (5) a lysine residue at the active site is postulated to form a Schiff base with the molecule which gives rise to the A side (acetic acid side chain) of PBG, whereas noncovalent bonding was assigned to the other δ-aminolevulinic acid, the source of the P side (proionic acid side chain). Recently a histidine residue has also been implicated to be essential for activity (6) and modification of 2 to 4 arginine residues from a total of 23 residues/monomer by phenylglyoxal or butanedione also inactivates PBG synthase.

It is known that thiol groups are essential for PBG synthase activity (2). Preparations of the enzyme are extremely sensitive to air oxidation and require the presence of thiol reducing agents for activity. Chemical modification by reagents directed toward alkylation of cysteine inactivate the enzyme, e.g. N-ethylmaleimide, iodoacetamide, p-chloromercuribenzoate (7), and iodoacetic acid (8, 9). Although many enzymes require free thiol groups for activity and are inactivated by cysteine alkylation (10-20), the precise role of these essential sulfhydryl groups is often not known. In some cases the function of the thiol group has been determined, e.g. glyceraldehyde-3-phosphate dehydrogenase (21, 22), or postulated, e.g. proline racemase (23).

Our studies on the alkylation of PBG synthase with IAc and IAM revealed differences in the characteristics of inactivation of the enzyme which raised the possibility that different
residues may be alkylated by these two reagents. This communication is concerned with the differential alkylation of specific cysteinyl residue(s), and their possible role in both the activation of PBG synthase and in a mechanism of PBG synthesis. The proposed mechanism postulates that these sulfhydryl group(s) may participate in acid/base catalysis necessary for the required protonation/deprotonation sequences.

MATERIALS AND METHODS

PBG synthase was purified from beef liver (24) and assayed as previously described (25). Protein was measured by the method of Lowry et al. (26) or modified to allow the determination in the presence of thiols (27). [1-\textsuperscript{14}C]IAm or [\textsuperscript{14}C]IAc were from New England Nuclear and extracted once with 
CCl4 prior to use. IAc was recrystallized from water; IAc, sodium salt, was recrystallized from water/ethanol. We thank Dr. D. Gurney of our laboratory for samples of enzymically synthesized PBG (28). Other chemicals were of reagent grade. New England Nuclear Aquasol was the scintillation fluid. Enzite trypsin was purchased from P-L Biochemicals, trypsin-TPCK was from Worthington Biochemicals. The amino acid was recrystallized from water; IAc, sodium salt, was recrystallized from water/ethanol. IAc-2H\textsubscript{2}O was recrystallized from water. Other chemicals were of reagent grade. New England Nuclear Aquasol was the scintillation fluid. Enzite trypsin was purchased from P-L Biochemicals. Trypsin-TPCK was from Worthington Biochemicals. The amino acid compositions were determined on a Duram amino acid analyzer after hydrolysis with 6 N HCl (29).

To experimentally alkylate the enzyme samples were pretreated with nonradioactive IAm or IAc, depending on the \textsuperscript{14}C-labeled alkylating agent subsequently used. This was carried out under active-site-protection conditions (i.e., no thiol added and in the presence of PBG). These conditions reduced the subsequent nonspecific incorporation of \textsuperscript{14}C radioactivity and did not alter the specific activity of the enzyme. Typically, 1.0 ml of PBG synthase (24 mg/ml) was dialyzed for 48 h against 3 x 1 liter of potassium phosphate buffer, pH 7.5, to remove all traces of exogenous thiol reducing agents. The enzyme was thoroughly dialyzed against potassium phosphate, pH 7.5, prior to subsequent \textsuperscript{14}C alkylation studies. These pretreated enzyme preparations were used in all experiments which subsequently involved the labeling of the enzyme with radioactive alkylating agents.

General Alkylation Method with IAm and IAc - The procedure for the alkylation of PBG synthase in the presence or absence of possible protecting agents was as follows. Usually, 1 ml of PBG synthase (4.8 \textmu M) in 100 mM potassium phosphate, pH 7.5, was activated by preincubation for 30 min with 20 \textmu M dithiothreitol. For experiments in which dithiothreitol was purposefully omitted, the enzyme was thoroughly dialyzed against potassium phosphate, pH 7.5, to remove all traces of exogenous thiol reducing agents. The alkylation reactions were started by the addition of various concentrations of IAm or IAc and performed both in the presence or absence of protecting agents and on activated and air-oxidized PBG synthase samples. Further details of the reaction conditions are given in the legends to Figs. 1, 2, and 6 to 8.

DTNB Protection against Inactivation by Alkylation - The following procedure was used in experiments in which the enzyme was treated with Ellman's reagent (30) (DTNB) prior to the alkylation. Each sample of 0.22 ml of PBG synthase (1.4 \textmu M) in 100 mM potassium phosphate, pH 7.5, 10 \textmu M dithiothreitol was preincubated at 37°C for 30 min. The activated enzyme was treated with DTNB (2.0 \textmu M) and after 30 min the excess reagents were removed by passage through a column of Sephadex G-25 (0.5 x 10.5 cm) equilibrated with 100 mM potassium phosphate, pH 7.5, containing no dithiothreitol. The excluded fractions containing the enzyme were collected under nitrogen and alkylated with IAm or IAc at a final concentration of 1.4 \textmu M. After incubation at 37°C for 30 min each sample was passed through a column of Sephadex G-25 (0.8 x 10.5 cm) equilibrated with the same buffer as above. The excluded fractions containing the enzyme modified with DTNB were incubated at 37°C for 15 min in the presence of 4 \textmu M dithiothreitol in order to release the thioredoxenase ion prior to the determination of the enzymic activity on 100-\mu l aliquots incubated for 15 min with 5 mM \textsuperscript{5}aminolevulinic acid.

Equilibrium Dialysis - To prepare alkylated samples of PBG synthase for equilibrium dialysis studies, 28 \mu M solutions of the enzyme in 100 mM phosphate buffer, pH 7.5, 2.0 \textmu M dithiothreitol were incubated with 5 mM IAm or 9 mM IAc for 60 min and extensively dialyzed against 50 mM phosphate buffer, pH 7.5. These preparations were at least 99.7% inactivated. The conditions for equilibrium dialysis using these alkylated preparations are given in the legend to Fig. 8.

Detection of Cys(Cm) from Alkylated PBG Synthase - Approximately 20 \mu M pretreated PBG synthase (1 ml) in 100 mM potassium phosphate, pH 7.5, 1.0 mM dithiothreitol was incubated at 37°C for 30 min before the addition of 1.5 \textmu M [1-\textsuperscript{14}C]IAm (final concentration) containing 1.5 x 10\textsuperscript{5} cpm of [\textsuperscript{14}C]/\mu M. After 20 min of incubation in the dark, under nitrogen, the solution was cooled to 2°C and made 5.0 mM to dithiothreitol and the enzyme was extensively dialyzed at 4°C to remove noncovalently bound alkylating agent. This procedure gave about 20% of the residual activity and contained 1.3 mol of the [\textsuperscript{14}C]-labeled alkylating agent bound/monomer of 35,000. Similarly, 1.5 mM [1-\textsuperscript{14}C]IAc (final concentration) containing 1.9 x 10\textsuperscript{5} cpm/\mu M gave a carboxymethylated PBG synthase sample with 10% residual activity and 0.8 mol of [\textsuperscript{14}C]-labeled alkylating agent bound/35,000.

The hydrolysis of 2-ml samples of alkylated PBG synthase was carried out in 0.1 N HCl at 110°C for 21 h (29) after the addition of 2 \mu M of nonradioactive Cys(Cm) to the sample. The HCl was removed by vacuum rotary evaporation at 40°C and the hydrolysates were reconstituted in 100 ml of 0.2 mM citrate/resuspending buffer, pH 2.2, prior to chromatography. Ascending thin layer chromatography on polygram cellulose MN 300 precoated sheets was performed with phenol/water, 4:1, v/w. The \textit{R}f for Cys(Cm) was 0.48 to 0.51. Paper electrophoresis on Whatman No. 3MM paper was performed in pyridine/acetic acid/water, 1:10:89, v/v, pH 3.6, on a Savant electrophoresis apparatus performed as described (28). The Cys(Cm) migrated 8 cm toward the anode. The papers were developed by spraying with ninhydrin solution (40 ml of ethanol, 10 ml of acetic acid, 4 ml of collidine plus 50 mg of ninhydrin) and the \textit{R}f values of the enzyme were determined both by enzymic activity measurements and \textsuperscript{14}C incorporation on 100-\mu l aliquots incubated for 15 min with 5 mM \textsuperscript{5}aminolevulinic acid.

Alkylation - Sodium acetate buffers (150 mM) were used in the range of pH 3.5 to 5.5 and potassium phosphate buffers (150 mM) were used for pH 5.8 to 8.0 in the following experiments. Acetate does not interfere with enzymic activity (as do citrate or malate buffer systems) nor did the changeover of the buffer system affect the alkylation. The pH did not change during the course of the reaction. The pH dependence of alkylation was determined both by enzymic activity measurements and \textsuperscript{14}C incorporation on each sample. Pretreated PBG synthase samples (0.5 \mu M, 6 \mu M) were activated by incubation with 2.0 mM dithiothreitol in the appropriate buffer. After 30 min at 37°C, IAm or IAc (final concentration of 0.4 mM) was added, each containing approximately 4.8 x 10\textsuperscript{5} cpm/\mu M. The control samples at several selected pH values contained 3.0 mM PBG, but no dithiothreitol. The reactions were continued for 20 min at 37°C cooled to 2°C and the solution was extensively dialyzed against 50 mM potassium phosphate, pH 6.8, 5 mM potassium phosphate, 95% methanol. The controls were dialyzed against a methanol containing buffer containing of the thioethanol. Samples of 50 \mu l were counted in 5 ml of Aquasol for 14C incorporation and also assayed.
for residual enzymic activity whereas 20-μl samples were assayed for protein content.

**Determination of Number of Residues Modified by Alkylation**

The preincubation enzyme (0.5 μl of a 11 μm solution in 100 mM potassium phosphate, pH 7.5) was activated with 2.0 mM dithiothreitol (final concentration) by preincubation at 37° for 30 min and then alkylated with [14C]IAm (or [14C]IAc) and further incubated at 37° for 30 min. The final concentration of IAc ranged from 0.036 to 2.3 mm and the final concentration of the IAc ranged from 0.07 to 4.7 mm. The specific activities for the various incubation mixtures with each of the specific alkylating agents were the same (approximately 106 cpm of 14C/μmol). The control experiments contained 0.15 mM, A; 1.0 mM, O; and 1.5 mM, n. B, inactivation by IAc: 9. At various times 10-μl aliquots were removed into 0.95 ml HCl. It can be concluded from Fig. 3, A, B, and C, that the extent of inactivation is dependent upon the concentration of the alkylating reagent but, as can be seen from the semilog plots, the rate of the irreversible inactivation deviates from linearity. This can be attributed to the simultaneous alkylation of the dithiothreitol continuously reducing the effective concentration of the alkylating reagents. Similar complications have previously been observed (36). Furthermore, as shown in Fig. 2, alkylation does not occur unless the enzyme is first reduced with dithiothreitol. Since, as shown below, the alkylation occurs on cysteinyl residues, the reduction by thiol reducing agents not only produces —SH groups essential for catalysis, but also exposes these groups for alkylation.

**Identification of Amino Acid Residue Modified by Alkylation**

Since the thiol activation of PBG synthase determines the susceptibility of the enzyme to inactivation by alkylation (Fig. 2), it was considered that cysteinyl residues may be the site(s) of modification. However, since under various conditions it is known that IAm and IAc can modify other residues such as histidine (37–40), methionine (41, 42), and glutamate (43), it was important to identify the residue(s) that were alkylated. As a preliminary indication of the nature of the residues that were alkylated, inactivation by IAm and IAc was carried out after DTNB had been added to a thiol-activated sample of PBG synthase (as described under "Materials and Methods"). The titration of the enzyme with DTNB fully protected the enzyme against inactivation by either IAm or IAc (Table D). This result strongly suggested that cysteinyl residues were alkylated.

To identify the modified residues conclusively, the enzyme was inactivated with [14C]IAm or [14C]IAc and hydrolyzed with HCl. It can be concluded from Fig. 3, A, B, and C, that both IAm and IAc alkylate only cysteinyl residues. Identical conclusions were obtained from results in all three systems.

**RESULTS**

**Inactivation by IAm and IAc**

PBG synthase requires thiol reducing agents for enzymic activity. Air-oxidized samples of PBG synthase have at most 0.002% of the specific activity of the enzyme activated with dithiothreitol. Consistent with this thiol requirement for activity is the observation that alkylation by IAm or IAc (8, 9) inactivates PBG synthase (Fig. 1, A and B). The extent of inactivation is dependent upon the concentration of the alkylating reagent but, as can be seen from the semilog plots, the rate of the irreversible inactivation deviates from linearity. This can be attributed to the simultaneous alkylation of the dithiothreitol continuously reducing the effective concentration of the alkylating reagents. Similar complications have previously been observed (36). Furthermore, as shown in Fig. 2, alkylation does not occur unless the enzyme is first reduced with dithiothreitol. Since, as shown below, the alkylation occurs on cysteinyl residues, the reduction by thiol reducing agents not only produces —SH groups essential for catalysis, but also exposes these groups for alkylation.
FIG. 2. Dependence of inactivation by alkylation upon thiol activation. PBG synthase was thoroughly dialyzed (3 x 2 liters) against potassium phosphate (100 mM, pH 6.8) to remove any thiols present after the purification procedure. Preincubations at 37° of 1.0-ml samples containing 1.3 mg (4.8 µM) of PBG synthase in 100 mM potassium phosphate (pH 6.8) with (▪, □) and without (○, △) dithiothreitol (DTT) (2.0 mM) were performed for 30 min and then IAm (0.6 mM, ○, △) or IAc (0.8 mM, □, △) was added at time zero. At t = 15 min, dithiothreitol (2.0 mM) was added to the incubation mixtures which initially had no thiol (○, □). At various times 10-µl aliquots were removed into 0.95 ml of standard assay medium at 2°. After 10 min of preincubation at 37°, the assay was started by the addition of &amanolevulinic acid (5.0 mM) and incubated for 15 min.

TABLE I

| Conditions of experiment | DTNB protection against inactivation by alkylation | Enzyme activity |
|--------------------------|-----------------------------------------------|-----------------|
|                          | DTNB  | IAc or IAm | IAc  | IAm  |
|                          | µmol PBG/h/mg of protein                       |
| -                        | -     | -          | -    | -    |
| +                        | -     | +          | 4.4  | 2.4  |
| +                        | +     | +          | 12.5 | 12.4 |

with samples of hydrolysates of the enzyme alkylated either with IAc or IAm. The alkylation of a cysteinyl residue of the synthase by IAc was recently reported (9).

Number of Cysteinyl Residues Modified — To determine the number of residues modified that lead to complete loss of enzymic activity, the degree of inactivation was measured as a function of the extent of alkylation. The extent of alkylation was determined by measuring the 14C incorporation following alkylation with [1-14C]IAm or [1-14C]IAc. The extent of the inactivation was varied either by using different concentrations of the reagents for a fixed time at constant pH or by alkylation with a fixed concentration of the reagent at different pH values. It can be seen from Fig. 4, A and B, that in each case the initial slope extrapolates to a value of 0.8 to 1.0 mol of the alkylation agent incorporated/monomer of 35,000. This implies that primarily one essential residue was modified in each case. (The slope deviated from linearity as inactivation exceeded about 80%.)

pH Dependence of Alkylation — The pH profile for alkylation was determined as a measure of the reactivity of the cysteinyl residues toward IAm and IAc. The reactivity was determined simultaneously by following both the loss of enzymic activity and the incorporation of 14C radioactivity at various pH values. The results given in Fig. 5, in which the residual activity is plotted against pH, demonstrate in both experiments that the inflection point occurred at pH 5.2. The experimental data obtained with IAm were in good agreement with a theoretical titration curve for a single ionizable group. The results of experiments with IAc were somewhat anomalous; at lower pH values some inactivation still occurred. This implied that other factors are interfering with the titration of a single ionizable group. However, these experiments illustrate that both reagents alkylate cysteinyl residues of high reactivity. Similar results were obtained in plots of pH versus 14C radioactivity incorporated.

Alkylation in Presence of Levulinic Acid or Porphobilinogen — A criterion for establishing whether a particular modification is active site directed is to determine whether the substrate or product protects against inactivation. It can be seen that the presence of PBG protected against the inactivation by either IAm or IAc (Fig. 6). This protection suggested that both IAm and IAc alkylated cysteinyl residues at the
Levulinic acid, a substrate analogue, is a competitive inhibitor of PBG synthase with a $K_i$ of $1.2 \times 10^{-4}$ M (5) which is similar to the $K_m$ for $\delta$-aminolevulinic acid. This analogue was used to avoid product formation and yet provide active site protection. The presence of levulinic acid in alkylation experiments uncovered a difference between the characteristics of inactivation by IAm and IAc. It can be seen from Fig. 7A that, whereas levulinic acid protected against the inactivation by IAc, it augmented the inactivation by IAm (Fig. 7B). This difference raised the possibility that the two reagents alkylate different cysteinyl residues of the primary sequence.

Although the alkylation by IAm was not protected by levulinic acid, the finding of protection by PBG suggested that the cysteinyl residue modified by IAm is near or at the active site which consists of two $\delta$-aminolevulinic acid binding sites, A and P (Scheme 1).

[14C]Levulinic Acid Equilibrium Binding to PBG Synthase — Since the alkylations of the enzyme by IAc and IAm were affected differently by levulinic acid, we investigated the binding characteristics of these alkylated derivatives of the enzyme and compared the results with those obtained with air-oxidized and thiol-reduced native enzyme. The binding of [14C]levulinic acid to both native and modified samples of PBG synthase was studied by equilibrium dialysis at 2°C. The modified PBG synthase samples used in these experiments had lost at least 99.7% of their original activity by alkylation. The results shown in Fig. 8 expressed as Scatchard plots (45) where $d$ is the number of moles of [14C]levulinic acid bound/mmol of 285,000. It can be seen in Fig. 8 and Table II that, whereas both alkylated samples of PBG synthase bound a maximum of 2 mol of levulinic acid the affinity of the enzyme alkylated with IAm for levulinic acid was approximately 3- to 4-fold greater than that of the enzyme alkylated with IAc. These results were very similar to those obtained with native thiol-reduced enzyme and native air-oxidized PBG synthase (Fig. 8 and Table II). It can be seen that the binding properties of the enzyme alkylated with IAm are similar to the native active enzyme, whereas the enzyme alkylated with IAc has similar properties to the air-oxidized preparation. It is, however, interesting to note that the inactivated samples of the enzyme bind the same maximum number

active site. The assay procedure described in the legend to Fig. 6 was necessary because of the inhibition of the enzymic activity by PBG (44).
FIG. 8. Binding of levulinic acid to native and alkylated PBG synthase as determined by equilibrium dialysis. The binding studies were done on air-oxidized and thiol-activated samples of native PBG synthase and on IAc-alkylated and IAm-alkylated samples of the enzyme. In each case the sample of enzyme (2.2 mg) with or without dithiothreitol (6.0 mM) in 0.5 ml of potassium phosphate (100 mM), pH 6.8, was placed in one chamber of the dialysis apparatus along with [14C]levulinic acid (1.6 x 10^5 cpm) whose final concentration ranged from 11.3 to 205 μM. To the second chamber the same buffer, with or without dithiothreitol, was added. The dialysis was performed at 4°C for 24 h and 5-μl aliquots were used for radioactivity determinations and 5-μl aliquots for protein and enzymic activity measurements. The alkylated samples were inactivated to approximately 99.7%. Native thiol-activated enzyme, □; native air-oxidized enzyme, ○; IAm-alkylated enzyme, △; IAc-alkylated enzyme, ●.

TABLE II
Equilibrium binding parameters of [14C]levulinic acid to PBG synthase

| Enzyme sample          | Maximum binding of levulinic acid | Binding constant |
|------------------------|-----------------------------------|-----------------|
|                         | mol/285,000 x 10^-5               |                 |
| Native, thiol-activated | 1.87                              | 1.1             |
| Native, air-oxidized    | 2.21                              | 7.1             |
| IAm-inactivated         | 1.77                              | 1.5             |
| IAc-inactivated         | 1.89                              | 4.8             |

a Binding constant = Kᵢ, intrinsic equilibrium dissociation constant calculated from the slopes of Fig. 8.

from the IAm-derived major 14C-labeled peptides. These differences could conceivably be attributed, in part, to a charge difference between the amide and carboxylate groups on the alkylated peptide. However, it can be seen from the fingerprint mapping carried out at pH 2.5 (Fig. 9, A and B) and at pH 6.5 (Fig. 9, C and D) that the different mobilities of the labeled peptides occurred by chromatography rather than by electrophoresis, and that the labeled enzyme tryptic digesta. Electrophoresis on this chromatogram was at pH 6.5.

The elution profiles obtained from similarly prepared tryptic digests of alkylated enzyme on cation exchange chromatography were also different (Fig. 10, A and B). Since the IAm peptide was eluted in a position corresponding to a more acidic peptide than the position of the IAc-labeled peptide, the elution position of the peptides labeled with IAm or IAc cannot be explained in terms of the difference in charge if the same peptide was alkylated.

Furthermore, the profiles of radioactive peptides obtained by molecular sieve chromatography of tryptic digests of [14C]IAm and [14C]IAc-labeled enzyme were again different (Fig. 11, A and B). The only significant radioactive peak from the IAc-labeled enzyme centered around Fraction 54 (Fractions 52 to 55), whereas the IAm-labeled enzyme produced three major radioactive fractions. Redigestion with trypsin of...
FIG. 10. PA-35 cation exchange resin chromatography of tryptic digests of [14C]IAc- (A) and [3H]IAm- (B) alkylated PBG synthase.

The column (0.6 × 19.0 cm) was operated at 50°C and 110 to 120 p.s.i. at a flow rate of 12 ml/h. Aliquots (50 µl) of the 1.3-ml fractions were used for the detection of peptides with fluorescamine (λex = 280 nm, λem = 480 nm, O---O). Aliquots (25 µl) were used for the detection of fractions which contained [14C] radioactivity (O- - -O). Conductivity in mho (m- - -m) was measured on fractions which contained no peptides. Peptides were eluted with a gradient of 200 ml of 0.05 M pyridine/ AcOH/water, pH 3.1, stirring and interconnected with 200 ml of 2.0 M pyridine/ AcOH/water, pH 5.1, applied at Fraction 10. In general the more acidic peptides are eluted earlier. A, 9.0 mg of tryptic peptides from [14C]IAc-alkylated PBG synthase containing 1.4 mol of [14C] per mol (35,000 daltons). The main radioactive IAc-derived peptide was in Fractions 39 to 41. B, 8.1 mg of tryptic peptides from [3H]IAm-alkylated PBG synthase containing 0.6 mol of [3H] per mol (35,000 daltons). The main radioactive IAm-derived peptides were in Fractions 7 to 16.

The latter larger pieces produced more of the radioactive peptide eluting in Fractions 79 to 84. The relative position of the fractions of the IAc-labeled peptide (Fractions 52 to 55) and the major IAm-labeled peptide (Fractions 79 to 84) were maintained on co-chromatography after redigestion with trypsin.

These data demonstrate that the peptides isolated after alkylation by IAm and IAc and tryptic digestion are indeed different. This conclusion was further supported by amino acid analysis of radioactive peptides isolated and purified by a combination of the above procedures. The peptide labeled with IAc was found to be an octapeptide with the following composition; Cys(Cm), Glx, Pro, Gly, Ala, Val, Tyr, Arg, whereas the major labeled peptide obtained from the enzyme alkylated with IAm was found to be a dipeptide consisting of Cys(Cm) and Tyr.

**DISCUSSION**

The results of this communication demonstrate that IAc and IAm each irreversibly inactivate PBG synthase by alkylation of highly reactive essential cysteiny! residue(s) probably located at the active site. Furthermore, we have shown that the inactivation by alkylation only occurs if the enzyme is initially reduced with thiol compounds; the state of cysteine reduction of PBG synthase is all important for activity. It has been claimed that thiol activation generates two cysteiny!
Thiol Groups in Porphobilinogen Synthase

The exquisite sensitivity of PBG synthase to inactivation by air oxidation, the protection by PBG against inactivation by IAc and IAm alkylation, and the possible discrimination by these alkylation agents between the two cysteinyl residues alkylated, all suggest that two cysteinyl residues are sufficiently close (~5 Å) for facile disulfide bond formation. It is possible to consider that this ready interconversion of —S—S and —SH groups is a means of physiological control of the enzymic activity. This could be mediated by the concentration of naturally occurring thiols or by NAD(P)H-dependent disulfide reductases (47).

Several enzymes are known which similarly contain a critical disulfide (48-52). Facets of the characteristics of PBG synthase outlined above have been observed for several other enzymes, e.g., thiol activation of lipoamide dehydrogenase generates two chemically nonequivalent cysteinyl residues (18, 53) and the two active site thiols of yeast alcohol dehydrogenase are somewhat discriminated by alkylation depending on both the nature of the alkylation reagent and the pH of the reaction (11). Glyceraldehyde-3-phosphate dehydrogenase has also some similar characteristics; discrimination of alkylation of the essential thiol group by IAc and IAm occurs on addition of NAD+ (54).

The different characteristics of the enzyme alkylated with IAc and IAm may be explained either by alkylation of the same —SH group carrying a different charge (—COO− or —CONH2), or by the fact that these reagents, by virtue of their charge difference, alkylate —SH groups on different cysteinyl residues. This is somewhat reminiscent of the discrimination of the alkylation of two histidinyl residues by IAc in ribonuclease (37-40). Consistent with the possible role of the charge difference directing alkylation is the observation that the ester of the substrate or the ester of the competitive inhibitor, levulinic acid, has a very poor affinity for the enzyme (5). It has been suggested that at the active site there exists a positively charged residue to orient the substrate (5). It would appear reasonable to assume that the IAc alkylates the —SH group in the vicinity of this charge group. The

Fig. 11. Molecular sieve chromatography of tryptic peptides of [14C]IAc- (A) or [14C]IAm (B)-alkylated PBG synthase. Radioactivity (●—●), (□—□) cpm/25 μl of 2.5-ml fractions (———). The major [14C]IAc-derived peptide was in Fractions 52 to 55 (A). The major [14C]IAm-derived peptides were in Fractions 79 to 83, 48 to 56, and 34 to 40 (B). The peptide Fractions 34 to 40, which correspond to larger sequences, on pooling and redigestion with trypsin-TPCK, yielded more of the radioactive smaller peptide eluting in Fractions 79 to 84. The relative elution positions of the radioactive IAc-derived peptide (A, Fractions 52 to 55) and the radioactive IAm-derived peptide (B, Fractions 79 to 84) were maintained after admixture, redigestion with trypsin-TPCK, and rechromatography.

Fig. 12. Proposed mechanism of porphobilinogen synthesis from 2 molecules of δ-aminolevulinic acid as catalyzed by the enzyme. A side, acetic acid side chain; P side, propionic acid side chain.
alkylation of PBG synthase by IAm is potentiated by levulinic acid. It has previously been observed that competitive inhibitors not only may protect against modification of a group at the active site, but may also enhance its alkylation by a possible "ligand"-induced conformational change (Ref. 55 and references therein).

With the demonstration that the -SH group(s) are probably at the active site, it seems reasonable to expect that these group(s) would be more reactive than those in normal cysteinyl residues. As a measure of the reactivity, the pH dependence of alkylation by IAm and IAc was investigated. With both reagents the inflection point of half-maximum reaction was at pH 5.2. This is 3 to 4 orders of magnitude lower than the pK<sub>a</sub> of nonactivated cysteinyl residues (55-57). The pK<sub>a</sub> values of essential cysteinyl residues in other enzymes have been estimated to vary between 5.0 and 9.2 (16, 20, 53, 55-60). The lower pK<sub>a</sub> values imply the stabilization of the thiolate anion. This may be achieved by thiol-base pair formation (−S<sup>−</sup>...H...S<sup>−</sup>) and has been suggested as a means of lowering the pK<sub>a</sub> of the essential cysteinyl residues in several other enzymes (53, 54, 58, 61, 62) and could play a role in the mechanism of PBG formation by PBG synthase. Since the alkylation rates were not measured above pH 7.5 because of competing alkylation of dithiothreitol, it is possible that the measured pK<sub>a</sub> values of I<sup>14</sup>B synthase are only the acid limbs of a bell-shaped pH-dependent profile as observed with papain (63, 64) and streptococcal proteinase (19).

In view of the different effects of levulinic acid on alkylation (protection against IAc, potentiation toward IAm inactivation) the different binding characteristics of the alkylated enzymes (reduced affinity after IAC alkylation, unaltered affinity after IAm alkylation), the similar protection by PBG toward alkylation by both reagents, the demonstration that different tryptic peptides are labeled by each reagent (by chromatographic and amino acid analysis), it would appear that IAc and IAm alkylate cysteinyl residues in different positions of the primary structure.

On examination of the composition of the octapeptide obtained from IAc alkylation and the dipeptide obtained from IAm alkylation, a rational explanation that the dipeptide is a subset of the octapeptide can be advanced if one assumed that the sequence at the carboxyl end of the octapeptide is -Arg-Tyr. For only the 0-dansyltyrosine was obtained from IAc alkylation and the dipeptide obtained from IAm alkylation, a rational explanation that the dipeptide is a subset of the octapeptide can be advanced if one assumed that the dipeptide is Cys(Tyr), for only the 0-dansyltyrosine was obtained from IAc alkylation and the dipeptide obtained from IAm alkylation, a rational explanation that the dipeptide is a subset of the octapeptide. This finding suggests that the dipeptide is not a subset of the octapeptide.

Although the occurrence of two Cys-Tyr sequences in one polypeptide may be considered to be rare, it is not without precedent occurring in bovine prothrombin, porcine phospholipase A2, and in several neurotoxins. In view, however, of some possible misgivings, the final decision regarding the alkylation of different cysteinyl residues by IAm and IAc must await further exploration. Experiments are in progress to elucidate this point (see "Note Added in Proof").

The mechanism of PBG formation as proposed by Nandi and Shemin (5) depicts an aldol condensation between one molecule of δ-aminolevulinic acid, which is in Schiff base linkage with the Λ site of the catalytic site, with a second molecule of δ-aminolevulinic acid at the P site. Inspection of this mechanism suggests the requirement for several enzymic protonation/deprotonation sequences catalyzed by particular residues at the active site. The finding that cysteinyl residue(s) at the active site are of high "reactivity" suggests that these essential residues may participate in the necessary acid/base catalysis by the enzyme. A similar acid/base role has been proposed for a cysteinyl residue in proline racemase (23) and histidine decarboxylase (33). In order to participate throughout the catalysis as depicted in Fig. 12, each group (B' or BH) must alternate its acid/base status. A convenient means of achieving this is to interpose an imidazole bridge (or even a water molecule<sup>6</sup>) for regeneration of the required state. Consistent with this suggestion is the observation that a histidinyl residue is essential for PBG synthase catalysis (6).

The mechanism in Fig. 12 involves the initial deprotonation, by B' (possibly a thiolate anion) of the δ-aminolevulinic acid molecule at the A site and the protonation of the carboxyl group by BH (possibly thiol) at the P site (Fig. 12, Step 1). The starting B' and BH status is regenerated by mediation of the imidazole bridge (Step 2). This is followed by a second protonation/deprotonation sequence with the elimination of water (Step 3). The subsequent transamination steps together with the mediation of the imidazole bridge system complete PBG formation and regenerate the initial state of the enzyme (Steps 4 to 7).

The mechanism in Fig. 12 is modified from that of Nandi and Shemin to incorporate the observation that the deprotonation of the immediate precursor of free PBG (Step 6) is stereospecific and, therefore, enzyme-catalyzed (68, 69). The important feature of our mechanism is the bridged system for thiol groups in Porphobilinogen Synthase 8973
Thiol Groups in Porphobilinogen Synthase

It seems that both reagents, at particular conditions, alkylate explained from our recent experiments in which the enzyme is uncertain (4).

Note Added in Proof—The ambiguity in part may be explained from our recent experiments in which there is a role of the metals in the catalysis for as yet their role that other variations on the precise roles of the groups alternation of the acid/base status as required. It is realized.

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Mechanism of porphobilinogen synthase. Possible role of essential thiol groups.
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