Dissection of the Early Steps in the Porphobilinogen Synthase Catalyzed Reaction

REQUIREMENTS FOR SCHIFF'S BASE FORMATION

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The porphobilinogen (PBG) synthase catalyzed reaction requires both Zn(II) and reducing equivalents for the production of PBG from two molecules of 5-aminolevulinic acid (ALA). An early step in the reaction is the production of a Schiff's base between PBG synthase and one ALA molecule. Because both substrate molecules are chemically identical, there had been no evidence of enzyme-catalyzed partial reactions of ALA. When the conditions where PBG is not formed, in this study, NaBH₄ was used to trap the Schiff's base formed between substrate ALA and active holo-PBG synthase, inactive apo-PBG synthase, and inactive methylmethanethiosulfonate-modified apo-PBG synthase. ALA-dependent NaBH₄ inactivation of these enzyme forms was quantitated at 50–62, 94–97, and 93–96% inactivation, respectively. [4-¹⁴C]ALA was used to determine the stoichiometry of Schiff's base trapping which was 2.3, 3.5–4.0, and 3.4 per octamer for holoenzyme, apoenzyme, and methylmethanethiosulfonate-modified apoenzyme, respectively. These results are consistent with four active sites per octamer or half-of-the-sites reactivity.

We conclude that the production of the Schiff's base formed between one ALA molecule and the enzyme requires neither Zn(II) nor reduced enzyme sulfhydryl groups. Furthermore, the possible number of kinetic schemes for formation of the quaternary complex of enzyme, Zn(II), and two ALA moieties, one as the Schiff's base forms between substrate ALA and PBG synthase at an active site lysine residue. Their conclusion, which has become an established part of the biochemical literature, was that the ALA involved in the Schiff's base becomes the A side of the product PBG.

Until recently, there had been some controversy concerning the requirement of Zn(II) for enzyme activity and the requisite stoichiometry of the zinc-enzyme complex. Since the PBG synthase catalyzed reaction is essential to the formation of porphyrin, chlorophyll, and corins, it has been widely investigated. However, because both substrate molecules are identical, there is difficulty in demonstrating intermediate reactions and the sequence of required bond breaking/making steps. Nandi and Shemin (1), working on the enzyme from the photosynthetic bacteria Rhodopseudomonas spheroides, demonstrated that a Schiff's base forms between ALA and PBG synthase at an active site lysine residue. Their conclusion, which has become an established part of the biochemical literature, was that the ALA involved in the Schiff's base becomes the A side of the product PBG.

Recently, Hasnain et al. (7), using extended x-ray absorption fine structure spectroscopy on human erythrocyte PBG synthase containing eight Zn(II) ions per octamer for full activity and that reduced sulfhydryl groups are required for the formation of a tight Zn(II) binding site. We also confirmed the work of Tsukamoto et al. (6) that enzyme purified in the presence of 10 μM Zn(II) is fully active and contains eight Zn(II) ions per octamer. Recently, Hasnain et al. (7), using extended x-ray absorption fine structure spectroscopy on human erythrocyte PBG synthase containing eight Zn(II) ions per octamer, established that the Zn(II) ion was coordinated to three sulfur atoms and that reduced sulfhydryl groups are required for the formation of a tight Zn(II) binding site. We also confirmed the work of Tsukamoto et al. (6) that enzyme purified in the presence of 10 μM Zn(II) is fully active and contains eight Zn(II) ions per octamer.
three per subunit results in complete loss of the intrinsic Zn(II) (4).

Through our work with MMTS-modified apo-PBG synthase, we have evidence that ALA is required for the formation of the tight Zn(II)-binding site (4). As Zn(II) binding precedes Zn(II) binding, we have evidence that the formation of product PBG, we can investigate the partial reactions which precede Zn(II) binding. The steps may include formation of the Schiff’s base, binding of the second ALA molecule, and/or formation of other chemical intermediates which precede PBG release from the enzyme. In the current work, we investigate Schiff’s base formation on holoenzyme, Zn(II)-free apoenzyme, and MMTS-modified apoenzyme under reaction conditions where formation of PBG either does or does not occur.

EXPERIMENTAL PROCEDURES

Materials—Aminolevulinic acid hydrochloride, TES buffer, 2-mercaptoethanol, dithioerythritol, KH₂PO₄, NaBH₄, and methylmethanethiosulfonate were purchased from Sigma. [4,5-³⁵S]Aminolevulinic acid (46 μCi/μmol) was purchased from Research Products International Corp. Aqueous counting scintillant was purchased from Amer sham Corp. Immobilized 8-hydroxyquinoline on Controlled-Pore Glass beads was purchased from Pierce Chemical Co. All other chemicals were reagent grade.

Enzyme Preparations—Preparation of bovine liver PBG synthase and preparation and characterization of MMTS-modified apo-PBG synthase were carried out as previously described (4). Fig. 2 summarizes the preparation and reconstitution of MMTS-modified PBG synthase.

Enzyme Assay—When it was imperative to control or determine Zn(II) concentrations in assay components, enzyme activity determinations were carried out in TES/KOH buffer treated to remove trace Zn(II) as previously described (4). Under most other conditions, standard 5-min 37 °C assays were carried out in 0.1 M potassium phosphate buffer (KP), pH 6.8, containing 10 mM dithioerythritol, 10 mM ALA, and 10 μM added ZnCl₂. Dithioerythritol is normally contaminated with substantial amounts of Zn(II) (8). Based on enzyme activity, we estimate that the total free Zn(II) under these assay conditions does not exceed 3 μM. Under conditions of low specific activity, assays were incubated for 20 min in order to ensure an Aₚₐ activity greater than 0.5 absorbance units (see Table I and “Results”). As ALA was not a substrate, activity is expressed as the production of a 1 μmol PBG/h.

Reaction of PBG Synthase, MMTS-modified Apo-PBG Synthase, and Apo-PBG Synthase with NaBH₄—in a total volume of 0.5 ml, 1–3 mg of PBG synthase (20–25 units/mg) were incubated in 0.1 M KP, pH 6.8, and 10 mM dithioerythritol at 37 °C for 10 min. The Zn(II)-containing holo-PBG synthase and reconstituted MMTS-modified apo-PBG synthase, the reaction mixture also contained 10 μM ZnCl₂ in addition to the Zn(II) introduced from the enzyme. In the case of apop-PBG synthase prepared from holo-PBG synthase or MMTS-modified apo-PBG synthase, the reaction mixture contained 10 mM EDTA in addition to the standard buffer components. In the case of MMTS-modified PBG synthase, dithioerythritol was left out of the reaction mixture.

Following the 10-min incubation, 0.5 ml of 0.1 M KP (without enzyme but containing the same additional reaction components as above) including 20 mM ALA was added. After 1 min at 37 °C, 50 μl of 1.0 M NaBH₄ were added, and the reaction mixture was placed in an ice-water bath. The reaction mixture remained on ice for 10 min with stirring. The pH was monitored and maintained at 6.8 by addition of 1 M acetic acid. The protein was then precipitated with addition of saturated ammonium sulfate to a final concentration of 60–75% saturation. After 30 min on ice, the reaction mixture was centrifuged; the supernatant was discarded and the protein pellet was redissolved in 0.5–1.0 ml of 0.1 M KP, pH 6.8, 10 mM dithioerythritol 10 μM ZnCl₂. The redissolved protein was dialyzed overnight against 500 ml of the same buffer. In order to control for non-ALA-dependent inactivation, parallel NaBH₄ reactions were carried out without the substrate ALA.

Analysis of PBG Synthase, MMTS-modified Apo-PBG Synthase, and Apo-PBG Synthase after Reaction with NaBH₄—During dialysis in the presence of both Zn(II)-containing mercaptoethanol, holo-PBG synthase was regenerated from all NaBH₄-treated enzyme samples. Following dialysis, protein concentrations were determined by UV absorption at 280 nm (Aₙ₅₀ = 1.13) (4). Parallel activity determinations, in KP, dithioerythritol buffer, were performed for the enzymes which had been treated with 1) NaBH₄ plus ALA, 2) NaBH₄ alone, and 3) enzyme (either holoenzyme or MMTS-modified apoenzyme) which had not been treated with NaBH₄.

³⁵S Labeling of PBG Synthase by Reaction of NaBH₄ in the Presence of [4,5-³⁵S]Aminolevulinic Acid—Reaction conditions were as described above with the addition of 0.5–3.0 μCi of [4,5-³⁵S]ALA per reaction. In these cases, radioactive ALA was added to the buffer, and aliquots were counted to determine the specific radioactivity of the ALA (0.05–0.3 μCi/μmol). The redissolved protein was dialyzed and analyzed for protein concentration, specific enzymatic activity, and specific radioactivity. ³⁵S-Samples were counted using a Packard Tri-Carb 460 Scintillation Counter working at >90% counting efficiency. Radioactive enzyme samples each contained in excess of 3000 cpm, equivalent volumes of dialysis buffer contained less than 200 cpm, and backgrounds were less than 50 cpm.

RESULTS

PBG Synthase Activity—Contained in Table I are the results of simple fixed time assays used to determine the assay conditions which would or would not support production of PBG by the various PBG synthase preparations.

Zn(II)-containing holo-PBG synthase, for which maximum activity is observed, can be produced in the following three ways: 1) holoenzyme in KP/dithioerythritol buffer, and 10 μM added ZnCl₂ (22 units/mg); 2) MMTS-modified apoenzyme in TES/mercaptoethanol buffer + Zn(II) (17 units/mg); and 3) MMTS-modified apoenzyme in KP/dithioerythritol buffer with or without additional Zn(II) (19 units/mg) (see also Fig. 2). When zinc-deficient or depleted enzyme is assayed in KP/dithioerythritol buffer, Zn(II) sufficient to stimulate maximum activity is introduced as a contaminant from the buffer components.
MMTS-modified apo-PBG synthase has lack of the putrid odor which normally occurs when the SCH, reagents because the methyl mercaptan modification is retained (0.0 unit/mg) (4). This is most easily verified by the or KPi buffers, neither of which contains any sulfhydryl modified apo-PBG synthase in TES/mercaptoethanol buffer synthase was shown by atomic absorption spectroscopy to holoenzyme in KPj dithioerythritol buffer with 10 concentration, i.e. 3.6 units/mg at 0.09 specific activity which can be reduced by raising the enzyme concentration. MMTS-modified apo-PBG synthase is inactive in either TES contain less than 0.1 zinc atom per octamer (4). The last method was not chosen to trap the Schiff’s base formed between enzyme and substrate because NaBH, Inactivation of and [4-14C]ALA synthesis being independent of metal ion, the the stoichiometry of four active sites per octamer. Again, the stoichiometry of labeling with [14C]ALA is about 3.5-4.0 per octamer. Again, the stoichiometry of four modifications yielding nearly inactive enzyme is consistent with four active sites per octomer. In the presence of EDTA and dithioerythritol, where both holo- and MMTS-modified apo-PBG synthase are inactive, NaBH, reduction in the presence of ALA results in enzyme stoichiometry of 2.3 14C-moieties per octomer yielding enzyme which is 90% inactivated is consistent with the proposal that octameric PBG synthase contains only four functional active sites or that PBG synthase exhibits half-of-the-sites reactivity (1, 2, 9, 10). The data presented can be interpreted with respect to 1) the requirements for Schiff’s base formation between ALA and PBG synthase, 2) the sequence of events which define the initial steps in the PBG synthase catalyzed reaction, and 3) the number of active sites per PBG synthase octamer. These subjects are discussed in turn below.

**Requirements for Schiff’s Base Formation**—The observation that NaBH, can be used to trap a Schiff’s base formed between [14C]ALA and PBG synthase under conditions where the lack of available Zn(II) precludes PBG formation demonstrates that the Zn(II) is not necessary for the formation of the Schiff’s base. Furthermore, because the Schiff’s base can be trapped on MMTS-modified enzyme, none of the essential sulfhydryl groups are involved in the steps preceding and including Schiff’s base formation. Unlike eucaryotic PBG synthase, bacterial enzymes from R. spheroides (11) and Rhodopseudomonas capsulata (12) are not inhibited by 10 mM EDTA but do proceed via a Schiff’s base mechanism. Although these data are consistent with Schiff’s base formation being independent of metal ion, the bacterial enzyme preparations were not analyzed for metal ion content. Therefore, the data remain open to interpretation.

**TABLE I**

| PBG synthase | Assay components | Specific activity |
|--------------|-----------------|-----------------|
| μM octamer   |                 |                 |
| MMTS-modified apo-enzyme |                 |                 |
| 0.094        | TES*            | 0.0             |
| 0.094        | TES, BME*       | 3.6             |
| 0.313        | TES, BME*       | 1.2             |
| 0.094        | TES, BME, Zn*   | 17.1            |
| 0.094        | KP*             | 0.0             |
| 0.094        | KP, DTE*        | 19.5            |
| 0.094        | KP, DTE, Zn*    | 19.1            |
| 0.094        | KP, IFTE, EDTA* | 0.3             |

| Holoenzyme   |                  |                 |
|--------------|-----------------|-----------------|
| 0.048        | KP*             | 5.7             |
| 0.048        | KP, DTE*        | 21.7            |
| 0.048        | KP, DTE, Zn*    | 21.7            |
| 0.048        | KP, DTE, EDTA*  | 0.6             |

*0.1 M TES-KOH, pH 7.0, where included, [mercaptoethanol] (BME) = 10 mM and [ZnCl2] (Zn) = 1 μM.  
*0.1 M KP, pH 6.8, where included, [dithioerythritol] (DTE) = 10 mM, [ZnCl2] = 10 μM, and [EDTA] = 10 mM.

Table I also illustrates that MMTS-modified apo-PBG synthase in TES/mercaptopethanol buffer exhibits moderate specific activity which can be reduced by raising the enzyme concentration, i.e. 3.6 units/mg at 0.09 μM octamer versus 1.2 units/mg at 0.31 μM octamer. We have previously shown that in TES/mercaptoethanol buffer, the specific activity of MMTS-modified apo-PBG synthase falls hyperbolically from 14 to 1.2 unit/mg as the octamer concentration is varied from 0.03 to 0.33 μM (4). This is an indication that the observed activity is a result of trace Zn(II) present in the assay mixture rather than from the enzyme preparation. Under all other assay conditions reported, enzyme specific activity is independent of enzyme concentration. MMTS-modified apo-PBG synthase was shown by atomic absorption spectroscopy to contain less than 0.1 zinc atom per octamer (4).

Inactive apo-PBG synthase can be produced by 1) placing holoenzyme in KP/dithioerythritol buffer with 10 mM EDTA (0.6 unit/mg), 2) using MMTS-modified apo-PBG synthase under the same conditions (0.3 unit/mg) or 3) with MMTS-modified apo-PBG synthase in TES/mercaptoethanol buffer at high enzyme concentrations (1.2 units/mg).

One other inactive form of the enzyme was investigated. MMTS-modified apo-PBG synthase is inactive in either TES or KP buffers, neither of which contains any sulfhydryl reagents because the methyl mercaptan modification is retained (0.0 unit/mg) (4). This is most easily verified by the lack of the putrid odor which normally occurs when the "SCH, modifications are removed by sulfhydryl reagents. Under these conditions, MMTS-modified apo-PBG synthase has little affinity for divalent metal ions (4).

**ALA-dependent NaBH4 Inactivation of and [4-14C]ALA Labeling of PBG Synthase**—PBG synthase was reacted with NaBH, in the presence and absence of ALA or [4-14C]ALA under five sets of conditions: two which support turnover (purified holoenzyme and holoenzyme prepared by reconstitution of MMTS-modified enzyme) and two which do not support turnover due to lack of Zn(II) (apo-enzyme prepared from holoenzyme or by reduction of MMTS-modified enzyme), and one which does not support turnover due to lack of Zn and modification of the sulfhydryl groups (MMTS-modified enzyme). In all of the above cases, treatment with NaBH, in the absence of ALA resulted in less than a 13% variation from the original activity. Under the NaBH4 reaction conditions at 0 °C, the time required for the production of one PBG molecule per active site (i.e. one turnover) is 2.5-3 min if there are four sites per octamer and 5–6 min if there are eight sites per octamer.

As presented in Table II, in the presence of Zn(II)-containing PBG synthase, NaBH4 reduction under turnover conditions results in PBG synthase at 9–11 units/mg, 50–62% of the initial activity of 20–26 units/mg. NaBH4 reduction in the absence of substrate ALA results in minimally inactivated PBG synthase with 19 units/mg. Under these conditions where PBG is being produced, reduction in the presence of [14C]ALA results in labeling with 0.28 14C-moieties per subunit. The stoichiometry of 2.3 14C-moieties per octamer yielding enzyme which is 90% inactivated is consistent with the proposal that octameric PBG synthase contains only four functional active sites or that PBG synthase exhibits half-of-the-sites reactivity (1, 2, 9, 10).
with respect to the involvement of metal ions in Schiff's base formation. Bacterial PBG synthase may be a Zn(II) metalloenzyme containing a more tightly bound Zn(II). There are numerous examples of Zn(II) metalloenzymes which are not inhibited by EDTA (13). A clear precedent is alcohol dehydrogenase for which the yeast enzyme is not inhibited by EDTA, whereas the liver enzyme is inhibited by EDTA. Both are indisputably Zn(II) metalloenzymes.

It is interesting to note that a greater proportion of the enzyme can be inactivated under non-turnover conditions than under turnover conditions. This difference, 50 versus 96% inactivation, serves as an assurance that the reaction conditions are sufficient to maintain the difference between holo- and apoenzyme. The difference may be explained on the basis that for Zn(II)-free enzyme, the reaction cannot proceed to product. Hence, the number of possible enzyme-bound intermediates is less than that for turnover conditions. Therefore, at any point in time, a larger proportion of the enzyme will exist as the Schiff's base and more Schiff's base will be trapped in the given 10-min reaction time.

In support of the rather unusual result that inactive PBG synthase will trap more of the Schiff's base than active enzyme, we refer to a short note by Chaudhry et al. (14) on alkylation of PBG synthase. Schiff's base trapping experiments were reported as preliminary results which "indicate that the fully active enzyme was able to incorporate labeled substrate in the presence of NaBH₄, but, as inactivation (by 2-iodoacetate) proceeded, the incorporation of labeled substrate was actually increased." These experiments were not quantitative and did not address the presence or absence of Zn(II). The stoichiometry of 2-iodoacetate labeling was later shown to be one per subunit with resulting loss of enzymatic activity (15, 16).

**Kinetic Mechanism**—Although it has been known that a Schiff's base is involved in the early steps in the reaction (1–3), one could have written at least 12 possible kinetic mechanisms for the initial steps. These kinetic mechanisms are illustrated in Fig. 3 and are numbered consecutively downward along the third kinetic event. They describe potential pathways toward the formation of a quaternary complex containing two ALA molecules, one as the Schiff's base, and the Zn(II) required for product formation. The kinetic schemes outlined in Fig. 3 are simplified by the constraints that 1) different conformational states of the enzyme are not considered, 2) all sulfhydryl groups are assumed to be reduced, and 3) we choose to follow the evidence of Jordan and Seehra (2) and Jordan and Gibbs (3) that the Schiff's base forms only to the ALA which becomes the F side of PBG.

Out of the 12 kinetic schemes presented in Fig. 3, only Schemes 7, 11, and 12 are the remaining possibilities. The current demonstration that Schiff's base formation can precede Zn(II) binding rules out Schemes 1–5 and 8–10 as mandatory kinetic schemes. To rule out Schemes 1 and 4–6, recall the strong evidence (2, 3) that the first ALA to bind at the active site is involved in Schiff's base formation. Although our current information cannot discriminate between Schemes 7, 11, and 12, we have reduced the number of possible kinetic schemes for formation of the quaternary complex from 12 to 3.

The three remaining kinetic schemes are not unique. They fix Zn(II) binding after Schiff's base formation, but also presume that Zn(II) binding precedes the formation of any bonds between the two ALA moieties. Although this need not be the case, it is appealing to consider the possibilities 1) that Zn(II) binding follows binding of the second ALA molecule and is accompanied by a conformational change which brings the two ALA moieties together as in Schemes 7 and 12 or 2) that Zn(II) binding may precede binding of the A site ALA and trigger a conformational change which creates the A site-binding pocket as in Scheme 11.

Kinetic Schemes 7, 11, and 12 also suggest that the mandatory kinetic sequence involves the binding and release of Zn(II) as part of the overall reaction mechanism. We have no additional data to support this hypothesis. In fact, the Zn(II) may remain bound, but only participate in a reaction step following Schiff's base formation. Possibly the Zn(II) ligands change during the course of the reaction, thus changing the Zn(II)/enzyme affinity. We have presented evidence that Zn(II) binds more tightly to enzyme in the presence of ALA (4). These interpretations which all imply a flexible environment around the Zn(II) are consistent with our (4) and Beyersmann and co-workers' (7) inability to locate an NMR

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**Table II**

| Initial enzyme* | NaBH₄ reaction components* | Supports turnover | Resulting specific activity | No NaBH₄ specific activity | Inactivation °C/octamer | units/mg | % |
|----------------|---------------------------|------------------|----------------------------|--------------------------|------------------------|---------|---|
| H              | DTE, Zn, [14C]ALA         | Yes              | 10.2                       | 26.5                     | 2.26                   | 62      | 2.26 |
| H              | DTE, Zn, ALA             | Yes              | 10.8                       | 21.4                     | 50                     | 50      | 50  |
| H              | DTE, Zn                  |                  | 18.7                       | 21.4                     | 13                     | 13      | 13  |
| MA             | DTE, Zn, [14C]ALA         | Yes              | 9.5                        | 21.3                     | 2.27                   | 55      | 2.27 |
| MA             | DTE, Zn, ALA             | Yes              | 9.1                        | 20.0                     | 55                     | 55      | 55  |
| MA             | DTE, Zn                  |                  | 17.5                       | 20.0                     | 55                     | 13      | 13  |
| H              | DTE, EDTA, [14C]ALA       | No               | 1.5                        | 24.9                     | 94                     | 94      | 94  |
| H              | DTE, EDTA, ALA           | No               | 1.1                        | 24.2                     | 96                     | 96      | 96  |
| H              | DTE, EDTA                |                  | 26.5                       | 24.2                     | -10                    | -10     | -10 |
| MA             | DTE, EDTA, [14C]ALA       | No               | 0.7                        | 21.3                     | 97                     | 97      | 97  |
| MA             | DTE, EDTA, ALA           | No               | 0.7                        | 21.2                     | 97                     | 97      | 97  |
| MA             | DTE, EDTA                |                  | 18.7                       | 21.2                     | 12                     | 12      | 12  |
| MA             | [14C]ALA                 | No               | 1.4                        | 20.9                     | 93                     | 93      | 93  |
| MA             | ALA                      | No               | 0.7                        | 20.5                     | 97                     | 97      | 97  |
| MA             | Buffer only              |                  | 18.6                       | 20.5                     | 9                     | 9       | 9   |

*H = holo-PBG synthase; MA, MMTS modified apo-PBG synthase.

*All reaction mixtures contained 0.1 M KPO₄, pH 6.8. Where included, [dithioerythritol] (DTE), [EDTA], and [ALA] = 10 mM and [ZnCl₂] (Zn) = 10 μM excess over subunits.
signal from \(^{113}\text{Cd}(II)\)-substituted PBG synthase.

On the Number of Active Sites—All of the results reported here are consistent with the stoichiometry of four active sites per octameric enzyme. In support of four active sites, Shemin, in a 1976 review (9), referring to the unpublished work of Shearer and Wu, reported that "it appears that out of the eight subunits, only four react with the substrate to form the Schiff's base." Other workers have addressed this question with ambiguous results. For instance, Seehra and Jordan (7) reporting on the inactivation of PBG synthase by 3- and 5-chloro[5,5-\(^{14}\)C]levulinic acid, stated, "the relationship between the loss of activity and the incorporation of radioactivity at low levels of inactivation suggested that the incorporation of 0.5 mol of alkylating agent/mol of subunit would lead to complete inactivation. However, in practice, the enzyme was fully inactivated only when 1 mol of reagent/mol of subunit has been incorporated... suggesting that half of the sites susceptible to alkylation are substantially more reactive than the others." In an elegant study of the inactivation of crude rat liver PBG synthase by 4,6-dioxoheptanoic acid (succinylacetone), Tschudy et al. (18) concluded, by comparison with purified enzyme from other sources, that the turnover number of the rat liver enzyme is consistent with eight active sites per octamer. Tschudy et al. include an excellent discussion of the complexities of half-of-the-sites reactivity on the bi-substrate enzyme PBG synthase.

Based on Shemin's report (9) that the stoichiometry of Schiff's base formation is four per octamer and their own evidence that octameric PBG synthase can be dissociated into functional dimers (19), Batlle et al. (20) proposed a mechanism whereby the active sites sat at the interfaces of "two kinds of subunits, that, although possibly having similar composition, play a different role in PBG synthesis." The proof of this hypothesis would lie in establishing the non-identity of the subunits. Wu et al. (21) rigorously demonstrated that bovine liver PBG synthase was an octamer composed of subunits which were indistinguishable by sedimentation equilibrium in 6 M guanidine HCl or by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Investigations of subunit composition by primary sequence analysis of bovine liver PBG synthase by Holberger (22) revealed that the NH\(_2\)-terminal sequence(s) was blocked and that the number of peptides on tryptic digest was equal to that predicted by the amino acid composition. We have confirmed that the NH\(_2\)-terminal sequences are blocked; however, there exists an intriguing report that only half of the NH\(_2\)-terminal residues of the bovine enzyme are acylated, suggesting a subtle difference in subunit structure (23).

In the absence of proof that the subunits are not identical, we are prompted to reflect of the meaning of half-of-the-sites reactivity (10). In this study, Schiff's base trapping under non-turnover conditions reproducibly results in the loss of all but about 3-7% activity concurrent with no more than four [4,\(^{14}\)C]ALA molecules trapped per octamer. The difference between 0% activity and 3-7% activity may simply reflect that small proportion of the four active sites which remains unmodified, or it may reflect the reactivity of the remaining "half-of-the-sites." These results are consistent with eight functional active sites (one per subunit), with only half of the sites being capable of catalyzing the reaction at a rate of 0.6 per s (at 37 °C) with the other four sites only capable of catalyzing the reaction at the rate of 0.02 per s. Alternatively, the second four sites may exhibit extremely high binding constant(s) for the substrate ALA (negative cooperativity).

Conclusion—The demonstration that Zn(II) is not necessary for the formation of Schiff's base is an important addition to our knowledge of the PBG synthase mechanism. This presents the first evidence of a partial reaction with the natural substrate ALA on PBG synthase under conditions which do not support turnover. Because the identity of the two substrate molecules has previously presented researchers with an all or nothing phenomenon, the trapping of Schiff's base on apoenzyme or MMTS modified apo-PBG synthase has opened the way toward investigating the partial reactions which may precede Zn(II) participation in the PBG synthase reaction.

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