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SYSTEMATIC SPECIFICITY STUDIES ON PORPHOBILINOGEN DEAMINASE

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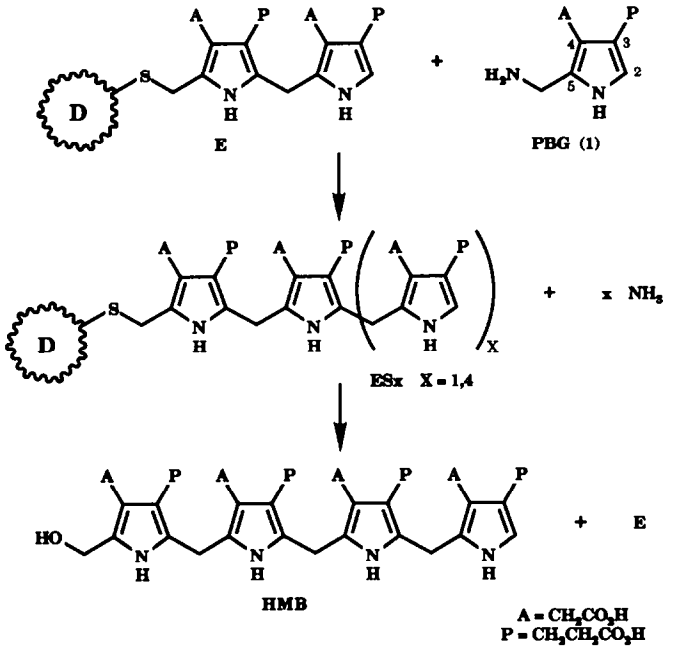
Abstract. Analogs of porphobilinogen (PBG) with different arrangements of the acidic side chains at C-3 and C-4 were tested for their ability to form complexes (ES_x, x = 1-4) with PBG deaminase (EC 4.3.1.8), the enzyme responsible for the tetramerization of PBG into hydroxymethylbilane (HMB). These studies show that an acidic side chain at C-4 in PBG is required for covalent interaction with the enzyme.

Porphobilinogen deaminase (Deaminase; EC 4.3.1.8) is the enzyme that carries out the head-to-tail condensation of porphobilinogen (PBG; **1**) with the release of hydroxymethylbilane (HMB) (Scheme I).[1] HMB is in turn the substrate for uroporphyrinogen III synthase (EC 4.2.1.75), the cyclizing enzyme responsible for the formation of uroporphyrinogen III, a key intermediate from which all the porphyrinoid pathways (heme, chlorophylls, vitamin B_{12}, etc) are derived.[2]

Scheme I

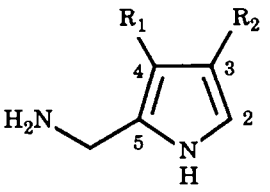


Deaminase (E; Scheme I) is a unique enzyme as it possesses a dipyrromethane cofactor derived from PBG as the attachment point for the first substrate molecule.[3] The stepwise tetramerization can be monitored by electrophoretic techniques as the different enzyme-substrate intermediates (ES_x, x = 1-4; Scheme I) are stable enough to be resolved.[4]

Over the years several PBG analogs have been analyzed as substrate/inhibitors of Deaminase.[5-7] These studies determined the disappearance of the pyrrole by the Ehrlich's assay,[8] or measured the production of uroporphyrin I (URO I; the chemical cyclization-oxidation product of HMB), but they did not reveal any information about the nature of the interaction with the enzyme.

A recent publication from our research group[9] initiated a more detailed study of the enzyme-substrate/inhibitor interactions in an attempt to determine the mechanism by which PBG reacts with Deaminase. Following the same philosophy but aiming at the functional requirements for recognition and binding of the pyrrolic units, nine analogs of PBG (**2-10**; Table 1) were synthesized,[10-11] and their ability to form ES_x complexes[12] analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE) and by fast protein liquid chromatography (FPLC).[4] Four of these compounds are correlated to the structure of PBG by the shortening (**2**), the extension (**3-4**), or the inversion (iso-PBG; **5**) of the acidic side chains. The rest correspond to the elimination of one (**6-9**) or both (**10**) acidic functions in PBG and its inverted analog, iso-PBG.

Table 1. PBG analogs and their ability to form ES_x[12] complexes with Deaminase.



	R_1	R_2	X in ES_x
(1)	A	P	1,2,3
(2)	A	A	1,2
(3)	A	B	1,2,3
(4)	P	P	1
(5)	P	A	1
(6)	A	H	1,2
(7)	H	P	-
(8)	H	A	-
(9)	P	Me	1
(10)	H	H	-

A = CH_2CO_2H P = $CH_2CH_2CO_2H$
B = $CH_2CH_2CH_2CO_2H$ Me = CH_3

On incubation with Deaminase none of the derivatives underwent catalytic turnover as determined by Ehrlich's assay (no consumption of PBG was observed), which showed the importance of both acidic side chains for the successful completion of the tetramerization and product release.

PAGE and FPLC analysis showed that the diacids (**2**), (**4**), and (**5**) formed ES_1 complexes with Deaminase after a short incubation period (15 min at 37°C), while (**3**) was incorporated more efficiently giving rise to a mixture of ES_2 and ES_3 complexes. Analog (**2**) also produced an ES_2 derivative after prolonged incubation (1h). In the case of the monoacidic compounds, complex formation was only detected with compounds (**6**) (ES_1 after 15 min; ES_2 after 1h incubation) and (**9**), which required 2h to completely transform the enzyme into a ES_1 complex (Table 1).

Since non-denaturing PAGE separates the different ES_X complexes by the number of charges, this technique could not be used in the case of aminomethyl pyrrole (**10**) because complexation between this compound and Deaminase would not increase the number of negative charges in the molecule. For that reason, [$H_2N^{13}CH_2$] pyrrole (**10**)[10] was incubated with Deaminase and analyzed by ^{13}C-NMR. The absence of any ^{13}C signal at ca. 25.1 ppm (py-$^{13}CH_2$-py)[13] indicated that no ES_X complex was formed.

Taken together, these results suggest that one of the requirements for covalent substrate binding is the presence of an acidic side chain at C-4 and that substitution at C-3 is not necessary, contrary to some earlier suggestions.[5]

An *in situ* activity assay on native gels[13] containing the above complexes provided interesting results. Iso-PBG (**5**) and the mono propionate (**9**) complexes were active toward PBG (URO I formation was observed upon incubation of the gel with PBG), in agreement with early reports[6] that described iso-PBG as a competitive inhibitor of Deaminase. It remains to be determined if the recovery in the activity was due to an exchange between PBG and the bound inhibitor (in a "classical" competitive fashion), or if the enzymatic machinery was able to incorporate three PBG molecules into the initial ES_1 complex (in spite of being unable to incorporate more than one molecule of inhibitor), releasing the corresponding HMB analog. Preincubation of Deaminase with (**9**) (2 h), followed by 15 min incubation with a same amount of PBG produced a mixture of ES_2 and ES_3 complexes (determined by PAGE) with no production of HMB (no formation of URO I was detected), which favours the latter mechanism for the recovery of the activity in the presence of a large excess of PBG (*in situ* activity assay conditions).

Diacetate (**2**), dipropionate (**4**) and monoacetate (**6**) ES_X complexes were inactive toward PBG (no URO I formation in *in situ* assay). The enzymatic formation of porphyrin in these assays may be blocked at either of two stages, substrate binding or hydrolysis of the product from the enzyme. The ES_1 and ES_2 complexes of (**2**) were separated by FPLC, incubated with an excess of PBG at 37°C for 15 min and subject to a second round of FPLC. The ES_1 complex was completely converted into ES_3, while the ES_2 complex was converted into a form that could not be recovered from the column, leaving open the possibility of the formation of the ES_4 complex, which has only been observed with the *E. coli* enzyme under conditions of electrospray MS.[14] This last result might indicate, at least in the case of the ES_2 derivative, that the complexation of the enzyme by the diacetate thwarts the mechanism by which Deaminase releases its product.

In conclusion, we have demonstrated the important role of the C-4 acidic side chain in the covalent binding of the substrate to the enzyme in the early stages of the PBG --> HMB transformation. The C-3 substituent, while still essential for the completion of the tetramerization, is not involved in the recognition and attachment of the first pyrrole molecule. Finally, the competitive-non competitive behaviour of the different inhibitors was explored and a rationale suggested for each case.

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10. The syntheses of the different analogs (**2**)-(**10**) were performed by the use of standard reactions, based on the directing effect of the substituents present in the pyrrolic intermediates and will be described elsewhere.
11. Analogs (**2**)-(**10**) were characterized by ^{1}H- and ^{13}C-NMR. Typical examples are:

    (**4**) (Ethyl methyl ester) ($CDCl_3$). ^{1}H-NMR δ 10.00 (br, 1H, N*H*); 6.45 (s, 1H, *H*-2); 4.85 (q, 2H, $CO_2CH_2CH_3$); 4.15 (s, 2H, CH_2NH_2); 3.52 (s, 3H, CO_2CH_3); 2.64, 2.60, 2.52, 2.47 (4t, 8H, 2 $CH_2CH_2CO_2R$); 1.18 (t, 3H, $CO_2CH_2CH_3$). ^{13}C-NMR δ 174.7; 173.3; 120.4; 119.8; 118.6; 60.3; 51.8; 34.9; 34.6; 34.3; 20.4; 18.7; 14.1.

    (**7**) (D_2O). ^{1}H-NMR δ 6.57, 6.05 (2s, 2H, *H*-2 and *H*-4); 3.96 (s, 2H, CH_2NH_2); 2.57, 2.28 (2t, 4H, $CH_2CH_2CO_2H$). ^{13}C-NMR δ 183.2; 123.8; 116.9; 108.8; 39.0; 36.0; 23.1.

    (**8**) (D_2O). ^{1}H-NMR δ 6.61, 6.04 (2s, 2H, *H*-2 and *H*-4); 3.96 (s, 2H, CH_2NH_2); 3.32 (s, 2H, CH_2CO_2H). ^{13}C-NMR δ 180.1; 124.9; 120.6; 117.9; 111.6; 37.5; 34.5.
12. For reasons of clarity we use the expression ES_X to denote the complexes of Deaminase with the PBG analogs even if they are not true substrates, i.e. do not produce HMB analogs as products of the reaction with Deaminase.
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