J. Enzyme Inhibition, 1990, Vol. 3, pp. 303–310
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PHOTODYNAMIC AND NON-PHOTODYNAMIC ACTION OF SEVERAL PORPHYRINS ON THE ACTIVITY OF SOME HEME-ENZYMES†

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(Received 3 April, 1989)

The action of porphyrins, uroporphyrin I and III (URO I and URO III), pentacarboxylic porphyrin I (PENTA I), coproporphyrin I and III (COPRO I and COPRO III), protoporphyrin IX (PROTO IX) and mesoporphyrin (MESO), on the activity of human erythrocytes δ-aminolevulinic acid dehydratase, porphobilinogenase, deaminase and uroporphyrinogen decarboxylase in the dark and under UV light was investigated. Both photoinactivation and light-independent inactivation was found in all four enzymes using URO I as sensitizer. URO III had a similar action as URO I on porphobilinogenase and deaminase and PROTO IX exerted equal effect as URO I on δ-aminolevulinic acid dehydratase and uroporphyrinogen decarboxylase. Photodynamic efficiency of the porphyrins was dependent on their molecular structure. Selective photodecomposition of enzymes by URO I, greater specificity of tumor uptake by URO I and enhanced porphyrin synthesis by tumors from δ-aminolevulic acid, with predominant formation of URO I, underline the possibility of using URO I in detection of malignant cells and photodynamic therapy.

KEY WORDS: Porphyrins, δ—aminolevulinic acid dehydratase, photooxidation, porphobilinogenase, uroporphyrinogen decarboxylase, erythrocyte deaminase.

INTRODUCTION

Activation of photosensitizers by light leads to photodegradation of proteins and nucleic acids.[1] In living cells, this process produces severe damage and might finally lead to cell inactivation and death.[2]

Porphyrins are known to very efficiently sensitize biomolecules, skin and malignant tumors,[3] and are becoming very important compounds in photo biology and photomedicine. Accumulation of porphyrins in cells might result from disturbed heme metabolism, due to either exogenous factors or genetic failure, leading to the characteristic skin photosensitization, which is the predominant symptom in cutaneous porphyrias. Certain porphyrins are retained more specifically and longer in tumors than in normal tissues when injected in humans and tumor-bearing animals. Subsequent illumination can destroy rather selectively the tumor, with only limited

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Abbreviations: Uroporphyrin I (URO I); uroporphyrin III (URO III); pentacarboxylic porphyrin I (PENTA I); coproporphyrin I (COPRO I); coproporphyrin III (COPRO III); protoporphyrin IX (PROTO IX); mesoporphyrin (MESO); δ-aminolevulinic acid dehydratase (ALA-D); porphobilinogenase (PBG-ase); uroporphyrinogen decarboxylase (URO-D).

damage to surrounding normal cells. This has become the basis of photodynamic therapy (PDT) for the treatment of solid tumors, although one limitation of this photochemotherapeutic technique is the induction of skin photosensitivity which might persist for 8–12 weeks after injection.[4]

For both the understanding of light dermatosis and for protecting the patients against photosensitization and so increasing the effectiveness of PDT, a better understanding of the underlying photodynamic processes is required. This objective has stimulated a large increase in basic research on porphyrin photobiochemistry over the past few years.

Controversy exists about the mechanism of photodynamic cell inactivation. It has been proposed that tissue damage is the result of lipid peroxidation[5] and complement activation,[6] but it is also important to consider that porphyrins can produce *in vitro* photoinactivation of enzymes.[7-11] It has been postulated that photodegradation of proteins can be the result of both photooxidation and photodynamic crosslinking leading to deterioration of cellular functions.[12]

Besides photodynamic inactivation we have recently described a novel light-independent effect of porphyrins on erythrocyte porphobilinogenase (PBG-ase) and deaminase[10] as well as aminolevulinate dehydratase (ALA-D).[11] The first report of these unusual properties of porphyrins was the potentiating action of hematoporphyrin on the horseradish peroxidase catalyzed oxidation of NADPH in the dark.[13,14] Much knowledge exists about the photosensitizing properties of porphyrins but little is known on their reactivity in the dark except the unique effect of uroporphyrin in stimulating collagen biosynthesis in human skin fibroblasts[15] and the activation of guanylate cyclase by protoporphyrin.[16] It is therefore important to establish whether porphyrins have biological effects other than their known photosensitizing properties and we have been conducting a series of studies on the possible cytotoxic and photodynamic action of porphyrins on several enzymes of the heme and other pathways.

We report here on the action of different porphyrins on the activity of human erythrocyte δ-aminolevulinic acid dehydratase (ALA-D), porphobilinogenase (PBG-ase), deaminase and uroporphyrinogen decarboxylase (URO-D) on incubation in the dark and under UV light.

MATERIALS AND METHODS

Uroporphyrin I (URO I) and coproporphyrin I (COPRO I) were kind gifts from the late Dr Torben With (Denmark). Uroporphyrin III (URO III), penta carboxylic porphyrin I (PENTA I), coproporphyrin III (COPRO III), protoporphyrin IX (PROTO IX) and mesoporphyrin (MESO) were obtained from Porphyrin Products – Logan, USA. All chemicals used were reagent grade.

Fresh human blood was obtained from the blood bank of San Carlos Hospital, Madrid, and erythrocyte hemolysates prepared as described elsewhere.[17] 10 ml of erythrocyte hemolysate were irradiated for 2 h at 37° C in Pyrex glass Petrie dishes of 40 mm diameter immersed in a water bath, in the presence of the test porphyrin (10 μM porphyrin for ALA-D, PBG-ase and deaminase or 100 μM porphyrin for URO-D). The final pH was adjusted to 7.0 with sodium phosphate buffer. A UV lamp (OSRAM, model HPW 125) with maximal emission at 365.5 nm was placed at 10 cm from the incubation vessels to obtain a light intensity of 40 W/m^2 measured at the

sample level. After irradiation the suspension was treated with Dowex 1-X8 (200–400 mesh) anionic resins, and then centrifuged for 10 min at 1000x g to remove the porphyrins added externally. Enzymic activities were then measured in the resulting systems under their corresponding standard incubation conditions.

ALA-D activity was measured as described by Batlle *et al.*;[18] PBG-ase and deaminase activities according to the method of Batlle *et al.*[17] and URO-D activity following the method of Afonso *et al.*[19] Controls in the presence of UV light and in the dark, with or without porphyrins were run in all experiments. The activity of the systems maintained in the dark and in the absence of porphyrin at 4°C was taken as 100%. Each data point represents the mean value of three experiments run in duplicates.

RESULTS AND DISCUSSION

Effect of Porphyrins on ALA-D

From previous experiments, it was found that photoinactivation of ALA-D by URO I and URO III was dependent on porphyrin concentration, time of irradiation and temperature (Afonso *et al.* unpublished results) and led to the choice of the conditions used here for assessing the response of ALA-D to the presence porphyrins under dark and light conditions.

Figure 1 shows the action of different porphyrins on the catalytic activity of ALA-D when treated for 2 h at 37°C with 10 μM of each. Photoinactivation occurred with all porphyrins. At the concentration tested URO I and PROTO IX caused the most (55%) while URO III and MESO the least (20%) photodecomposition. PENTA I was also a rather effective photoinactivation (50%) agent.

Histidine residues are located at the active site of ALA-D[20] so that photooxidation of this aminoacid is very likely the cause of photoinactivation of the enzyme in the presence of the porphyrin photosensitizers. It has been shown that the efficiency of porphyrins in sensitizing the photooxidation of aminoacids was dependent upon the nature of the side chains which protrude from the tetrapyrrole core.[21] This previous conclusion would justify the different effect of the different porphyrins observed here

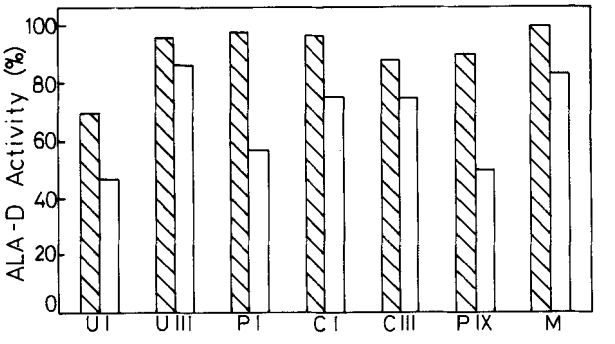

FIGURE 1 Effect of different porphyrins on the activity of erythrocyte ALA-D, in the light (□) and in the dark (▨). Final concentration of all porphyrins tested in the reaction mixture was 10 μM, temperature was 37°C and irradiation time 2 h. The activity of the controls without porphyrin, kept for 2 h at 37°C was; in the dark, 100%; in the light, 96%. U I, URO I; U III, URO III; P I, PENTA I; C I, COPRO I; C III, COPRO III; P IX, PROTO IX; M, MESO. Other experimental conditions were as indicated in the text.

but from the present results it might also be added that besides the nature of the substituents, their specific and relative localization in the ring is important since it can be seen that the effects of the URO I and URO III isomers are quite different. Therefore, photodynamic effectiveness of the porphyrin dyes is strikingly dependent both on the type and distribution of the side chains attached to the beta carbons of the cyclic tetrapyrrole.

Another interesting finding is that URO I was as effective as PROTO IX in photodegradating ALA-D. It has previously been suggested that photodynamic damage differs with the porphyrin species and with the target structure, PROTO being rather noxious in hydrophobic environment.[21] We could then explain the strong action of PROTO IX on ALA-D as being due to the hydrophobic nature of its active center which provides further support to the postulated mechanism of action for this enzyme.[22]

In the dark, only URO I significantly reduced ALA-D activity (about 30%) and practically no changes were produced by the other porphyrins (Figure 1). This effect is noticeable and indicates that URO I has some unique property which makes it rather selective for certain aminoacids. We have previously proposed that the light-independent inactivating action of uroporphyrins on PBG-ase and deaminase could be due to direct inhibition of the enzyme by induced conformational changes at their active site through binding of the porphyrin to the protein.[10] It could be expected that the dark action of URO I on ALA-D might be attributable to a similar mechanism yet clearly different from simple competitive inhibition of the enzyme by URO since, as indicated in methods, porphyrin was removed from the system before measuring enzyme activity. This assumption gains support from the observation that non-photodynamic inactivation of this enzyme is protected by its substrate δ-aminolevulinic acid (ALA) (Afonso *et al.*, unpublished results).

Effect of Porphyrins on PBG-ase and Deaminase

Previous results[10] have shown that URO I and URO III produce significant light-dependent and light-independent inactivation of PBG-ase and deaminase. At a concentration of 10 μM, 37°C and after 2 h incubation, the activity of both enzymes was reduced by 60–80%. These conditions were selected for comparatively assessing the action of the porphyrins under light and darkness. The results obtained (Figures 2 and

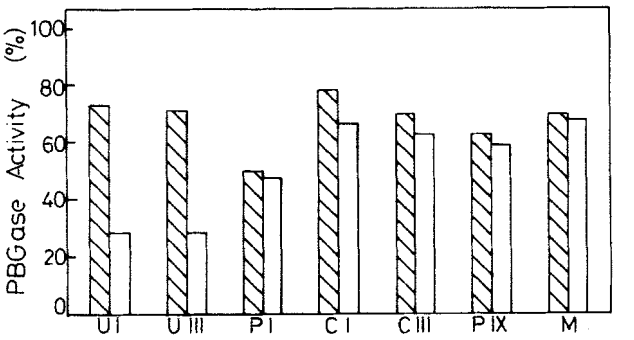

FIGURE 2 Effect of different porphyrins on the activity of human erythrocyte PBG-ase in the light (□) and in the dark (▨). All other conditions, abbreviations and controls levels as in legend to Figure 1.

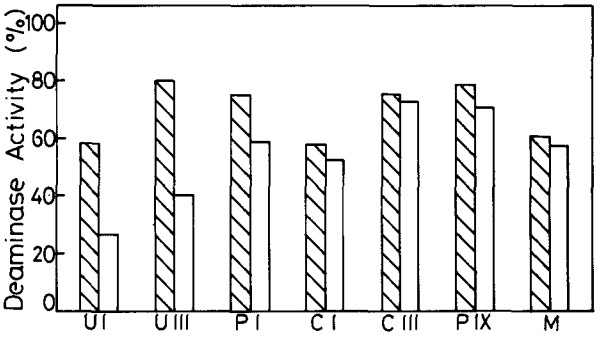

FIGURE 3 Effect of different porphyrins on the activity of human erythrocyte deaminase in the light (□) and in the dark (▨). All other conditions, abbreviations and controls levels as in legend to Figure 1.

3) show the expected strong photooxidation of both enzymes using either URO I or URO III as sensitizers; URO I was slightly more powerful than URO III on deaminase. In the dark, URO I and URO III also significantly reduced both activities.

The other porphyrins tested diminished PBG-ase and deaminase activities both in darkness and light, but it should be noted that photoinactivation was much lower than non-photodynamic inactivation, and in some cases nil. This indicates that the action of these other porphyrins on enzyme activities is mainly due to attachment of the tetrapyrrole to the protein at or near the active site competing with either the substrate, or polypyrrol intermediates or even the products of the reaction, acting therefore more as inhibitors than as sensitizers. The loss of action of PROTO IX as sensitizer could also suggest that the environment of the active center of these enzymes is hydrophilic in nature.

URO I and URO III produced both photooxidation and dark inactivation to a great extent. The latter effect could also be attributed to changes in the conformation around the substrate binding site while photodegradation is most likely to be caused by oxidation of essential aminoacid residues such as histidine, and secondary inter and/or intramolecular crosslinking with free amino groups. Electrophoretic analysis of photodecomposition products of PBG-ase and deaminase showed extensive crosslinking (data not shown). The strong photosensitizing properties of URO I and URO III would also support the hypothesis for a more hydrophilic active site in these enzymes.

Effect of Porphyrins on URO-D

It has been demonstrated that URO I produced photooxidation of URO-D,[9] an action which was dependent on porphyrin concentration, time and temperature of incubation. However only a significant photoinactivation was attained for 100 μM URO, i.e. 10 times the concentration found to be effective on ALA-D, PBG-ase and deaminase. This indicates that URO-D might have a more tightly folded inflexible conformation which makes it more resistent to dye-sensitized photochemical modifications.

Examination of the dark and light effect of other porphyrins besides URO I on URO-D activity was studied at the higher concentration of 100 μM. The results (Figure 4) show that URO I and PROTO IX caused the most photo-decomposition,

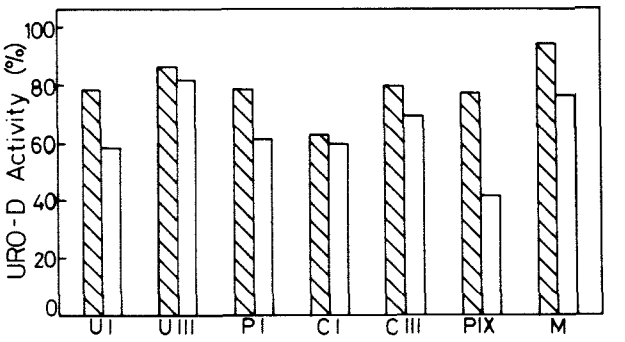

FIGURE 4 Effect of different porphyrins on the activity of human erythrocytes URO-D in the light (□) and in the dark (▨). Final concentration of all porphyrins tested in the reaction mixture was 100 μM, temperature was 30°C and irradiation time 2 h. The activity of controls, without porphyrin, kept for 2 h at 30°C was; in the dark, 100%; in the light, 98%. All other conditions and abbreviations as in legend to Figure 1.

rather low photooxidation occurring in the presence of the other porphyrins, including URO III, where some 10–40% light-independent inactivation occurred.

To explain the effect of photodegradation of URO-D due to URO I and PROTO IX the same reasoning applied to ALA-D is applicable. Histidyl residues are essential for URO-D activity and the presence of one or two lysines at the active site has also been visualized[23] so that photooxidation of imidazole rings followed by secondary inter and/or intrapeptide crosslinking would lead to enzyme inactivation. Electrophoretic analysis of photodegradation products of URO-D, indicating the occurrence of crosslinking, support this view (data not shown). These results also suggest that besides a possible tightly folded protein conformation, the active center of URO-D might be of a hydrophobic nature, because, as already mentioned, PROTO IX usually exerts the most extensive changes on hydrophobic environments. As to the significant non-photodynamic inactivation provoked by URO I and PROTO IX, direct blocking and structural modification of the protein may occur by analogy with the natural substrate of the reaction.

CONCLUSIONS

In summary, light-dependent and light-independent inactivation of ALA-D, PBG-ase, deaminase and URO-D has been found to occur by the action of URO I whereas in both ALA-D and URO-D, PROTO IX also produced photodynamic and non-photodynamic inactivation and for PBG-ase and deaminase, URO III equalled the action of URO I.

Photodecomposition of the enzymes has been attributed to photooxidation of histidine residues, followed by crosslinking; whereas the dark effect was considered to be the result of direct inhibition of the enzyme.

In ALA-D and URO-D, the existence of a hydrophobic active center has been inferred while that of PBG-ase and deaminase appears to be more hydrophilic.

It has also been observed that peripheral porphyrin substitution markedly affects the rate of aminoacid photooxidation. Such notable dependence of photodynamic effectiveness of porphyrins on their molecular structure could open new possibilities

in the field of photochemical modification of aminoacids in proteins and could also be of potential usefulness in gaining knowledge of their tertiary structures, as well as to provide information about the location and identification of essential aminoacid residues.

These results on the dark and light action of several porphyrins, confirmed previous findings about the effect of URO I on some heme enzymes and are of relevance regarding an understanding of the mechanism of photosensitization produced by porphyrins, as well as in the known general context of the citotoxicity produced by their accumulation in cells and tissues of porphyric patients and patients receiving PDT.

It is worth emphasizing that we have found that URO I is the most powerful photosensitizer of all porphyrins tested and its photooxidation action was significant on all the enzymes studied and this finding has been reflected in studies with other enzymes of both the heme and other pathways (Afonso *et al.*; unpublished results) as well as a mixture of glutathion-S-transferases, a heme-binding Z protein, a cytochrome P_{450} isozyme and hemopexin.[8]

Moreover, free or liposome encapsulated URO I uptake was selectively higher by tumors, when added to explants tissue cultures of breast adenocarcinoma tissues and other organs (Paredes *et al.*; unpublished results). It was also found that porphyrin biosynthesis from free and encapsulated ALA, added to explant tissue cultures was greatly enhanced in tumors as compared to liver, skin, kidney and brain with a skin/tumor porphyrin concentration ratio of only 0.2 and the predominant porphyrin formed was again uroporphyrin.[24] Consequently, selective and high photodecomposition of enzymes by URO I, greater specificity of tumor uptake for URO I and enhanced URO I synthesis by tumor explants from ALA, are results that assign to URO I a relevant role in connection with PDT, and further studies should be carried out to establish its potential clinical application in the diagnosis and phototherapy of cancer as already proposed. [25]

Acknowledgements

Alcira M. del C. Batlle holds the post of Principal Scientific Researcher in the Argentine National Research Council (CONICET). Susana G. Afonso is a Research Assistant at CONICET and thanks the Instituto de Cooperación Iberoamericana (ICI), Spain, for an external fellowship. The support of CONICET, University of Buenos Aires from Argentine and the CAICYT (PB 85-0015), FISss (89/0024), ICI from Spain is gratefully acknowledged. Alcira M. del C. Batlle is also grateful to Ministerio de Educación y Ciencia (MEC), Spain and the Association for International Cancer Research (AICR), U.K. for special help.

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