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FURTHER EVIDENCE ON THE PHOTODYNAMIC AND THE NOVEL NON-PHOTODYNAMIC INACTIVATION OF UROPORPHYRINOGEN DECARBOXYLASE BY UROPORPHYRIN I†

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The action of uroporphyrin I (URO I) on the activity of red cell uroporphyrinogen decarboxylase (URO-D) in the dark and under UV light was studied. Light-dependent-and light-independent inactivation was observed. Both effects increased at increasing concentrations of URO I, the former reached its maximum at 150 μM of sensitizer. At 100 μM of URO I, both light and dark inactivation were temperature dependent amounting to about 50% at 30–37°C. The velocity of dark inactivation increased with increasing temperature in the range of 0 to 45°C. Photoinactivation can be ascribed to primary oxidation of essential aminoacids, very likely histidyl residues, followed by secondary inter or intrapeptide cross-linking. Dark inactivation could be the result of both oxidation and cross-linking (although to a less degree than that produced by light) and also direct inhibition of the enzyme by induced conformational changes at its active site through binding of the porphyrin to the protein. When the action of URO I was tested on partially purified URO-D, the enzyme appeared to be more susceptible to the dark than to the light effect.

KEY WORDS: Uroporphyrinogen decarboxylase, uroporphyrinogen I, photodynamic – non-photodynamic inactivation, porphyrins.

ABBREVIATIONS: ALA-D, δ-aminolevulinic acid dehydratase: COPRO I, coproporphyrin I: COPRO III, coproporhyrin III: PBGase, porphobilinogenase: PCT, Porphyria Cutanea Tarda: PDT, photodynamic therapy: PROTO IX, protoporphyrin IX: RBC red blood cells: URO I, uroporphyrin I: URO III, uroporphyrin III: URO-D, uroporphyrinogen decarboxylase.

INTRODUCTION

Accumulation of porphyrins in cells as a result of disturbed heme metabolism or their injection in human or animals leads to the characteristic skin photosensitization, which is the hallmark in cutaneous porphyria. The discrimination of porphyrins between normal and malignant cells, their specific and much longer retention by the latter and their photosensitizing properties, allowing both detection and then selective destruction of the tumor with only limited or negligible damage to surrounding normal cells, are the basis of photodynamic therapy (PDT) for the treatment of solid tumors.[1]

†To the memory of Dr. Torben K. With.

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However, it is also known today, that porphyrins have biological effects other than their well known role as photosensitizers.[2,3] One of these properties is their reactivity in the dark, which acquires great importance in connection with the known general toxicity produced when porphyrins accumulate in cells and tissues of porphyric patients and those under PDT.

We have observed that porphyrins photo-inactivated URO-D and in an attempt to explain the mechanism of light dermatosis in Porphyria Cutanea Tarda (PCT) we proposed that porphyrins could play the dual role of inhibiting and photoinactivating the enzyme in the skin, extending its photodynamic action of lysosomal damage.[7] More recently we described a novel dark effect of porphyrins on the activity of some heme-enzymes. We have found that besides the expected photodegradation, porphyrins, particularly uroporphyrin I (URO I), provoked inactivation of δ-aminolevulinic acid dehydratase (ALA-D), porphobilinogenase (PBGase), deaminase and uroporphyrinogen decarboxylase (URO-D)[4,5,6] when the enzymes were incubated in its presence in the dark.

It was also found that both the light and the dark effect of porphyrins on the activity of the enzymes was porphyrin concentration-, time- and temperature dependent, and while 50% or higher inactivation was produced by 10 μM URO I on ALA-D, PBGase and deaminase[4] after 2 h incubation at 37°C, 10 times more URO I was needed to obtain the same effect on URO-D,[6,7] suggesting that URO-D might have a more tightly folded inflexible protein conformation and so become more resistant to the porphyrin induced chemical modifications. It was considered therefore that examination of the action of porphyrins on the activity of the enzymes, might cast some light on their molecular structure and native configuration.

We report here further results on the effects of URO I on the activity of URO-D of human red blood cells (RBC) when incubated in the dark and under UV light.

EXPERIMENTAL PROCEDURE

Fresh human blood was obtained from the blood bank of San Carlos Hospital, Madrid, and erythrocyte hemolysates prepared as described elsewhere.[8] Erythrocyte hemolysates (10 ml) were irradiated in Pirex glass Petri dishes (40 mm d) immersed in a water bath, in the presence of porphyrin. 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 10 cm from the incubation vessels to obtain a light intensity of 40 W/m2 measured at the sample level. On a sunny day with sun altitude 70, the light intensity in UV-A is approximately 45 W/m2, and light intensity reaching the dermis under these conditions[9] should be about 20 W/m2. After irradiation the suspension was treated with Dowex 1-X8 (200–400 mesh) anionic resin, and then centrifuged (1,000 × g) for 10 min to remove the porphyrins added externally. The enzymic activity of URO-D was then measured in the resulting system under the corresponding standard conditions as described by Batlle *et al.*[7] Controls in the presence of UV light and in the dark, with and without porphyrins were run in all experiments; the activity of the systems incubated in the dark and in the absence of porphyrins was taken as 100%. Dark and total inactivation were measured as indicated above. Light inactivation was calculated as the difference between total inactivation and dark inactivation. Each figure represents the mean values of three different experiments run in duplicates.

URO I and coproporphyrin I (COPRO I) were kind gifts of the late Dr Torben K. With (Denmark). Other porphyrins (uroporphyrin III (URO III), coproporphyrin III (COPRO III), and protoporphyrin IX (PROTO IX)) were obtained from Porphyrin Products (Logan, USA). All chemicals used were reagent grade.

Partial purification of URO-D was carried out as follows. 400 Human RBC (400 ml) were hemolyzed and the hemoglobin was removed by treating the suspension of RBC in sodium phosphate buffer 0.003 M pH 7.4 (1 : 3, v : v) with DEAE cellulose, in batch, as described by Llambias and Batlle.[8] The enzyme was eluted from the resin by washing (3 × 90 ml) of the same buffer (0.134 M) and centrifuging (1,000 × g) for 15 min. Supernatants were collected and ammonium sulphate fractionation was performed. The fraction precipitating between 50–75% saturation containing URO-D activity was separated, suspended in a small volume of Tris-HCl buffer (0.05 M) pH 7.4 and chromatographed in a Sephadex G-100 column, according to the method of Koopmann *et al.*[10] A preparation of URO-D purified about 330 fold was thus obtained, and referred to as partially purified URO-D to compare with the so-called crude URO-D (hemolyzed RBC). All manipulations were conducted at 4°C.

Electrophoretic analysis was performed as described by Laemmli *et al.*[11]

RESULTS AND DISCUSSION

Effect of URO I Concentration

In human PCT either hereditary or sporadic and in its animal model (hexachlorobenzene-induced porphyria) excessive accumulation and excretion of uroporphyrins, most belonging to series I, is due to hepatic hypoactivity of URO-D.[12]

Besides PCT, in Congenital Erythropoietic Porphyria, URO I is the predominant porphyrin. Selective and high photodecomposition and light-independent inactivation of enzymes[6] by URO I, greater specificity of tumor uptake for URO I and increased URO I biosynthesis by tumor explants from δ-aminolevulinic acid[13] and the unique dark effect of URO I stimulating collagen synthesis in cultured human fibroblasts[2] indeed assign to this porphyrin a relevant biological role. So we have extended our studies on the light and dark effects of porphyrins on URO-D using URO I.

We had found[7] that significant photoinactivation of URO-D was only attained using 100 μM URO I, a concentration 10 times that producing similar effects on ALA-D, PBGase or deaminase.[4,5] Here we have examined the action of different concentrations of URO I, keeping both exposure time and incubation temperature fixed, on the catalytic activity of RBC crude URO-D, in the presence and absence of UV light.

It can be seen in Figure 1 that URO I produced noticeable inactivation of the enzyme, and this effect occurs in the presence or in the absence of light. Both photodynamic and non-photodynamic action were dependent on URO I concentration; however while linear for total and dark inactivation, photooxidation appears to respond in a non-linear fashion and reaches its maximum at 150 μM.

We have already reported on the light-independent inactivation of some heme-enzymes by different porphyrins and it is of interest that Lim and Gigli[14] have also shown that high concentrations of PROTO IX decreased the activity of the C5 component of guinea pig complement in the dark, supporting further the possibility that porphyrins do possess additional biological effects which are independent

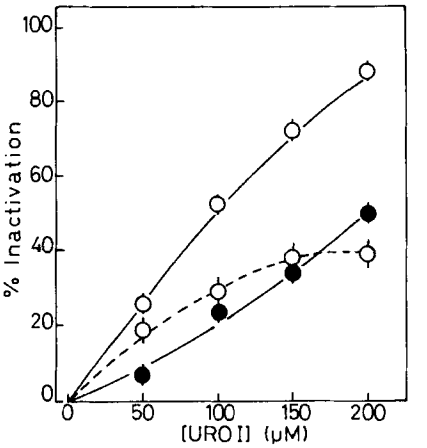

FIGURE 1 Effect of different concentrations of URO I on RBC URO-D activity. Exposure time was for 2 h, and temperature 37°C. (○---○) light, (●—●) dark, (○—○) total inactivation. Other experimental conditions as indicated in the text.

of light. These effects could be important in understanding the mechanism of their known cytotoxicity produced when they accumulate in cells and tissues of patients with cutaneous porphyrias and those under PDT.

The total *in vitro* inactivation of URO-D in the light, summing up photodynamic and non-photodynamic action at high concentration is very important, nearly 90% at 200 μM, of which half or more than half is due to the dark effect; only at the lowest concentration tested (50 μM) was photooxidation higher than dark inactivation. While much knowledge exists concerning the photosensitizing properties of porphyrins not much is known as to their reactivity in the absence of light. Photoinactivation of URO-D can be ascribed to photodegradation of its histidyl residues located at the active site; photooxidation of the imidazole ring followed by secondary inter-or intrapeptide cross-linking would lead to decreased enzyme activity.[15,16] Electrophoretic analysis (Figure 2) of photodegradation products of purified URO-D indicated the occurrence of cross-linking, supporting the above view.

However, unexpectedly, electrophoresis also showed the appearance of aggregated proteins when treatment with URO I was carried out in darkness, suggesting that covalent cross-linking also occurred. It has been proposed that non-photodynamic inactivation of the enzyme could be attributed to direct inhibition and structural modification of the protein, mainly due, in the case of URO-D, by analogy with the natural substrate of the reaction.[6] It appears now that dark inactivation can also be partly mediated by covalent cross-linking, although to a smaller extent than that observed under UV light. It is possible, then, that in the presence of porphyrins a reducing agent such as glutathione or cysteine and molecular oxygen, even in the dark, generates a reactive oxygen species which leads to oxidative degradation of the enzyme. This proposal would find support in earlier observations of Aft and Mueller[17] who showed that hemin effective degradation of specific proteins and glutathione reductase can be achieved in the presence of oxygen and 2-mercaptoethanol or NADPH; components required for producing the reactive oxygen species. In either case, light or dark, enzyme inactivation resulting from extensive cross-linking is dependent on the presence of oxygen.[17-21]

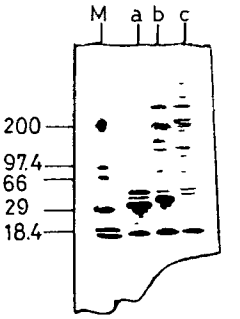

FIGURE 2 Electrophoretic analysis of purified URO-D products resulting from 100 μM URO I treatment. Line M: molecular weight markers (200, M_r = 200,000 (β-amylase); 97.4, M_r = 97,400 (phosphorylase B); 66, M_r = 66,000 (bovine serum albumin); 29, M_r = 29,000 (carbonic anhydrase); 18.4, M_r = 18,400 (β-lactoglobulin)). Line a: URO-D alone, in the dark. Line b: URO-D in the presence of 100 μM URO I in the dark. Line c: URO-D in the presence of 100 μM URO I under UV light. Other experimental conditions as indicated in the text.

Effect of Temperature and Time of Exposure to URO I

It has been found[7] that photoinactivation was dependent on incubation temperature reaching 50% or more at 30°C. Here, studies were extended to examine the effect of temperature on URO-D activity in the presence of URO I, in either light or darkness. Figure 3 illustrates results obtained at 30 and 37°C. It is observed that total inactivation is again 50% or higher at the temperatures shown, confirming the previous findings.

However, while photoinactivation seems to be more or less the same magnitude at 30 and 37°C, dark inactivation and so total inactivation increased with increasing temperature in the range of 0–45°C (data not shown). These findings suggest that under UV light, uroporphyrin initially causes photooxidation of the protein, a reaction known to be temperature independent, followed by a cross-linking reaction which can still proceed in the dark.[22] In the dark, inactivation at 30°C is lower than at 37°C; it is known that interpeptide cross-linking given the conditions, can still occur in darkness, but is significantly decreased at lower temperatures.[16] The present results

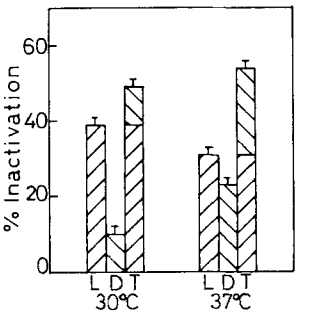

FIGURE 3 Effect of temperature on URO-D activity. Exposure time was for 2 h and concentration of URO I was 100 μM. (▨) light inactivation (L); (▧) dark inactivation (D); (▩) total inactivation (T). Other experimental conditions as indicated in the text.

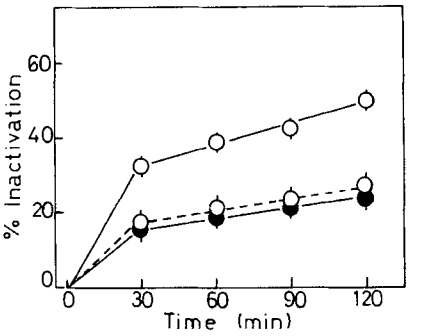

FIGURE 4 Effect of exposure time to URO I on URO-D activity. URO I concentration was 100 μM and temperature 30°C. (O- - -O) light inactivation, (●—●) dark inactivation, (O—O) total inactivation. Other experimental conditions as indicated in the text.

therefore confirm that in aerobiosis, porphyrins (or rather URO I) provokes both light-dependent and light-independent inactivation of URO-D. Under both conditions oxidation is the primary effect, followed by secondary cross-linking although to a different degree depending on the lighting conditions.

The influence of time of exposure of the enzyme to URO I (Figure 4) was now tested under dark and UV irradiation, but at 30°C, for up to 2 hours. Both light and dark inactivation contributed almost equally to total inactivation, which increased with time and was about 50%. These changes can be related to enhanced protein uptake of URO I with time and are in good agreement with earlier work.[4,7,23]

Effect of Different Porphyrins

It had been found[4,5] and here confirmed (Figure 5) that significant inactivation of URO-D by URO I was only attained at concentrations above 50–100 μM, i.e., 5–10 times concentrations found to be equally effective on ALA-D, PBGase and deaminase. This would be indicative of URO-D having a more tightly folded inflexible molecular

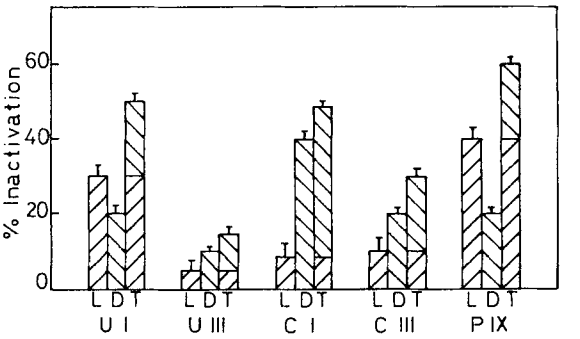

FIGURE 5 Effect of different porphyrins on the activity of URO-D. Final concentration of all porphyrins tested in the reaction mixture was 100 μM, temperature was 30°C and time of exposure was 2 h. (▨) light inactivation (L); (▧) dark inactivation (D); (▩) total inactivation (T). UI = URO I; UIII = URO III; CI = COPRO I; CIII = COPRO III; PIX = PROTO IX. Other experimental conditions as indicated in the text.

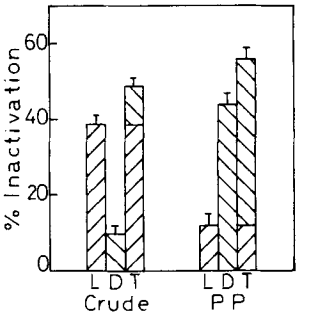

FIGURE 6 Effect of URO I on the activity of crude and partially purified (PP) URO-D. Concentration of URO I was 100 μM, temperature was 30°C and time of exposure was 2 h. (▨) light inactivation (L); (▩) dark inactivation (D); (▩) total inactivation (T). Other experimental conditions as indicated in the text.

conformation. The action of different porphyrins on URO-D activity was separately tested at a concentration of 100 μM for 2 hours at 30°C. Under these conditions URO I and PROTO IX caused the most photoinactivation (30–40%), while only 5–10% occurred in the presence of URO III, COPRO I and COPRO III. Light-independent inactivation was 40% with COPRO I and 30% with URO I, COPRO III and PROTO IX.

The particularly high susceptibility of URO-D to the action of URO I and PROTO IX can be explained on the basis of the existence of histidyl and lysine residues at the active site of the enzyme, so that primary oxidation of the imidazole rings by certain oxygen species in either light or dark would be followed by secondary inter or intrapeptide cross-linking leading to enzyme inactivation. In the dark URO I is very likely attaching to the enzyme at or near the active center, changing the local conformation around the substrate binding site, and therefore reducing its activity. In addition, the strong action of URO-D of PROTO IX, known to be rather noxious in hydrophobic environments[24], would suggest that the active site of this enzyme is more hydrophobic in nature.

Effect of URO I on Crude and Partially Purified URO-D

Total inactivation of partially purified URO-D under UV light (Figure 6) was slightly higher than that of crude preparations. However when the effect of light and dark were analyzed separately, unexpectedly, photoinactivation was rather low when using the purified enzyme and the dark effect was greater instead. It might be that in purified URO-D the active site is more exposed to the porphyrin or less protected than in crude preparations and so the effect of inactivation by direct blocking and modifying its conformation is enhanced over that of oxidation of essential histidyl residues and secondary cross-linking.

CONCLUSIONS

These studies provide additional information as to the photosensitizing action of porphyrins, particularly URO I on URO-D, confirming that the phototoxic effect can

be associated with light-induced inactivation of proteins due to primary oxidation of essential aminoacid residues and secondary cross-linking.

The existence of a very important dark action of porphyrins on enzyme activity, already observed for other heme and non-heme enzymes and confirmed here, indicates that porphyrins possess previously unknown biological effects that are independent of their photosensitizing properties. This novel dark effect of URO I and PROTO IX (and others) might account for the known general toxicity of porphyrins due to their accumulation in cells and tissues of porphyric patients and those undergoing PDT, as well as other abnormalities like the sclerodermatous lesions occurring in light-protected body areas of patients with PCT and Erythropoietic Protoporphyria.

The mechanism of this damage by porphyrins in the dark is not yet elucidated but can be ascribed to a combination of both oxidation and cross-linking and direct inhibition of the enzyme by induced structural changes at its active site due to binding of the porphyrin to the protein; all of these reactions leading to inactivation and/or denaturation or destruction of proteins.

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References

1. Kessel, D. (1984) *Photochem. Photobiol.*, **39,** 851.
2. Varigos, G., Schiltz, J.R. and Bickers, D.R. (1982) *J. Clin. Invest.*, **69,** 129.
3. Ignarro, L.J., Wood, K.S. and Wolin, M.S. (1982) *Proc. Nat. Acad. Sci. USA*, **79,** 2870.
4. Afonso, S.G., Chinarro, S., Muñoz, J.J., Salamanca, R.E. and Batlle, A.M. del C. (1987) *Boll. Ist. Dermatol. S. Gallicano*, **XIII,** 35.
5. Stella, A.M., Parera, V.E. and Batlle, A.M. del C. (1989) *Rev. Arg. Derm.*, **70,** 209.
6. Afonso, S.G., Chinarro, S., Muñoz, J.J., Salamanca, R.E. and Batlle, A.M. del C. (1990) *J. Enz. Inhibit.*, **3,** 303.
7. Batlle, A.M. del C., Salamanca, R.E., Chinarro, S., Afonso, S.G. and Stella, A.M. (1986) *Int. J. Biochem.*, **2,** 143.
8. Llambias, E.B.C. and Batlle, A.M. del C. (1971) *Biochim. Biophys. Acta*, **227,** 180.
9. Bickers, D.R., Pathak, M.A. and Magnus, I.A. (1979) *Dermatology in General Medicine*, pp. 942–994. New York; McGraw-Hill.
10. Koopman, G.E., Juknat de Geralnik, A.A. and Batlle, A.M. del C. (1986) *Int. J. Biochem.*, **18,** 935.
11. Laemmli, U.K. (1970) *Nature (Lond.)*, **227,** 680.
12. Batlle, A.M. del C., Salamanca, R.E., Magnin, P.H., Afonso, S.G., Chinarro, S., Stella, A.M., Wider, E.A. and Lenczner, J.M. (1985) *Rev. Arg. Derm.*, **66,** 245.
13. Fukuda, H., Paredes, S. and Batlle, A.M. del C. (1989) *Drug Design and Delivery*, **5,** 133.
14. Lim, H.W. and Gigli, I. (1981) *J. Invest. Dermatol.*, **76,** 4.
15. van Steveninck, J. and Dubbelman, T.M.A.R. (1984) *Biochim. Biophys. Acta*, **791,** 98.
16. van Steveninck, J., Dubbelman, T.M.A.R. and Verweij, H. (1983) (D. Kessel and T.V. Dougherty (Eds)) *Porphyrin Photosensitization*, pp. 227–240. New York; Plenum Press.
17. Aft, R.L. and Mueller, G.C. (1985) *Life Sci.*, **36,** 2153.
18. Sandberg, S. and Romslo, I. (1980) *Biochim. Biophys. Acta*, **593,** 187.
19. Sandberg, S. and Romslo, I. (1982) *Photobiochem. Photobiophys.*, **4,** 95.
20. Bickers, D.R., Dixit, R. and Mukhtar, H. (1982) *Biochem. Biophys. Res. Commun.*, **108,** 1032.

21. Afonso, S.G. *et al.* (1990) unpublished observations.
22. Dubbelman, T.M.A.R., Haasnot, C. and van Steveninck, J. (1980) *Biochim. Biophys. Acta*, **601,** 220.
23. Vincent, S.H., Holeman, B., Cully, B.C. and Muller-Eberhard, U. (1986) *Life Sci.*, **38,** 365.
24. Sandberg, S. and Romslo, I. (1981) *Clin. Chim. Acta*, **109,** 192.