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CYTOSOLIC AND MITOCHONDRIAL ENZYMES INACTIVATION BY UROPORPHYRIN IN LIGHT AND DARKNESS*

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The effect of uroporphyrin I (UI) on several cytosolic and mitochondrial enzymes (succinyl CoA synthetase, δ-aminolevulinic acid synthetase, rhodanese, lactate dehydrogenase) has been examined. All the enzymes were inactivated in the presence of the porphyrin both in the dark and under UV light.

KEY WORDS: Uroporphyrin, δ-aminolevulinic acid synthetase, succinyl CoA synthetase, rhodanese, lactate dehydrogenase, glutamate dehydrogenase

INTRODUCTION

The photodynamic action of sensitizers requires the presence of oxygen and is mediated by photooxidation of biomolecules in the cells.[1] It has been demonstrated that a number of amino acids such as cysteine, methionine, histidine, tyrosine and tryptophan, are potential substrates for the oxidative dye-photosensitized reactions.[2]

Porphyrins are known to very efficiently sensitize biomolecules in the skin and malignant tumors[3] and so, they play an important role in photobiology and photomedicine. Accumulation of porphyrins in cells as a result of either alterations of the heme pathway or after their exogenous administration, leads to the characteristic skin photosensitization, which is the main symptom in cutaneous porphyrias and in patients under photodynamic therapy (PDT).

Porphyrin photodamage has been mainly ascribed to lipid peroxidation.[4–6] However, it has been demonstrated that tetrapyrroles and related compounds can also provoke inactivation of proteins.[7–9] Rapid inactivation of lysozyme[10] and yeast alcohol dehydrogenase[11] occurred upon illumination in the presence of hematoporphyrin.

* Dedicated to Professor Dr. A.O.M. Stoppani on his 80th birthday.

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Hematoporphyrin derivatives have variable photosensitizing effects on several mitochondrial and cytosolic enzymes from rat mammary adenocarcinoma.[12] Incubation of yeast glutathione reductase in the presence of hemin destroys enzyme activity.[13] The presence of protoporphyrin in cultures of human neutrophils photoinactivated succinate dehydrogenase, glutamate dehydrogenase (GLDH), lactate dehydrogenase (LDH) and acid phosphatase.[14]

We have reported that light inactivation of four cytosolic heme enzymes, δ-aminolevulinic acid dehydratase (ALA-D), porphobilinogenase (PBGase), deaminase and uroporphyrinogen decarboxylase (URO-D) from human erythrocytes[15–18] is dependent on uroporphyrin I (UI) concentration, temperature and time of exposure of the protein to the porphyrin, as well as the presence of oxygen. Light-independent inactivation by UI was also observed in the dark, and this effect is of importance when porphyric patients and those under PDT are being considered.

To establish whether these light and dark effects of UI are a more general phenomena, we have extended our studies to examine the action of UI on other cytosolic and mitochondrial enzymes such as δ-aminolevulinic acid synthetase (ALA-S), succinyl CoA synthetase (Succ.CoA-S), rhodanese, LDH and GLDH.

MATERIALS AND METHODS

Enzyme Sources

Bovine liver rhodanese (EC 2.8.1.1) was obtained from Sigma; rabbit muscle LDH (EC 1.1.1.28) and bovine liver GLDH (EC 1.4.1.3) from Boehringer Mannheim GmbH. Succ.CoA-S was obtained from a crude sonicated extract of *Rp. palustris*,[19] and ALA-S from a crude homogenate of mouse liver.[20]

Enzyme Preparation

Succ.CoA-S (10 mg/ml) and ALA-S (10 mg/ml) were resuspended in 0.5 M Tris-HCl buffer pH 7.2. Rhodanese (0.1 mg/ml) was solubilized in 0.05 M Tris-HCl buffer pH 8.7; LDH (0.1 mg/ml) in 0.01 M sodium phosphate buffer pH 7.2 and GLDH (0.15 mg/ml) in 0.1 M potassium phosphate buffer pH 7.2.

Enzyme Treatment

Enzyme solutions (5 ml each) were placed in Pyrex glass Petri dishes (50 mm diameter) in the presence of 10 μM free UI and immersed in a water bath maintained at either 37°C or 30°C. A UV lamp (OSRAM, HPW125) was placed 10 cm from the incubation vessels to obtain a light intensity of 40 W/m^2 measured at the sample level. Solutions incubated in the dark were placed in the same water bath and protected from light by means of aluminium foil. Controls in the absence of porphyrins were treated in the same way. Once the period of exposure to UI was completed the suspensions were treated with Dowex 1-X8 resin to remove the added porphyrin, then centrifuged at 1,000 × g for 10 min. Enzyme activities were measured in the resulting supernatants. The activity of systems maintained in the dark and in the absence of porphyrin at 4°C was taken as 100%. Each data point represents

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the mean ± SD of five plates run in duplicate experiments. Student's t-test was used for statistical analysis of the data.

Enzyme Activities

Succ.CoA-S activity was measured following the method of Wider de Xifra and Tigier,[21] ALA-S activity as described by Lombardo *et al.*,[22] rhodanese activity according to Sörbo,[23] LDH as described by Kornberg[24] and GLDH according to Strecker.[25]

Enzyme Units and Specific Activities

One unit of Succ.CoA-S, ALA-S or rhodanese activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product under the standard conditions. One unit of LDH or GLDH activity is defined as the amount of enzyme that produces a diminution of 0.01 units of absorbance at 340 nm per minute. Specific activities are expressed as units per milligram of protein.

RESULTS AND DISCUSSION

Succinyl CoA Synthetase

Succ.CoA-S activity was unchanged when maintained for 2 h at 37°C in the dark or under UV light. The presence of UI in the dark reduced the activity by 25%, and irradiation resulted in 80% inactivation (Figure 1A).

Bacterial Succ.CoA-S is a tetramer $\alpha 2\beta 2$, its active site is located at the contact region between the α and β subunits.[26–28] The first reaction to occur involves phosphorylation of a histidine residue in an α subunit.[28] The enzyme contains significant amounts of tyrosine and tryptophan[30] and one of these tryptophan residues on a β subunit acts as a "signal group" for the attachment of the substrates to the protein; the modification of this residue impairs the catalytic reaction. Also in a β subunit an essential sulphydryl group is located close to the CoA binding site[27] and its photooxidation inactivates the enzyme.[30]

It is clear then, that a number of essential amino acids could be the target of UI action, either by their reaction with the porphyrin resulting in their alteration in the dark, or by their further photooxidation under UV light.

δ-Aminolevulinic Acid Synthetase

Because ALA-S is a labile enzyme, treatment with or without UI was for only 30 minutes at 30°C in the dark or under UV light.

In the dark, in the absence of UI, the enzyme activity was unchanged. Under UV light or in the dark in the presence of UI, ALA-S activity decreased by 70%. Irradiation in the presence of the porphyrin inactivated further the enzyme (Figure 1B).

ALA-S has a lysine residue at its active site which binds the cofactor pyridoxal phosphate (PPy).[31] It has been shown that at room temperature and under UV light, PPy and Tris buffer react forming a Schiff's base-like product.[32] In darkness, this reaction is minimal,

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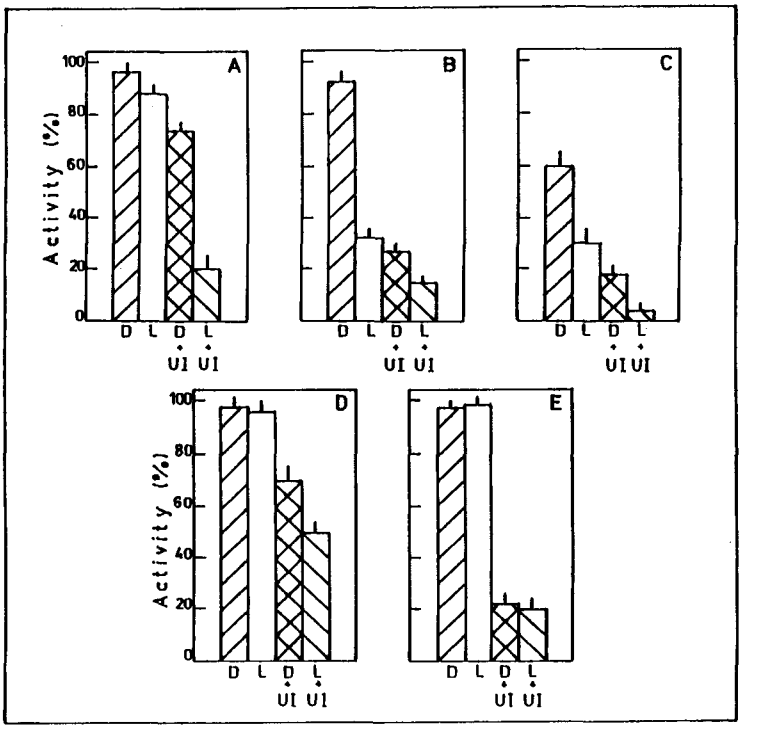

FIGURE 1 Effect of UI on Succ.CoA-S (A), ALA-S (B), rhodanese (C), LDH (D) and GLDH (E). The enzymes were treated in the dark (▨, ▩) or under UV light (□, ▧) in the presence (▧, ▩) or in the absence (▨, □) of 10 μM UI, during 2 h at 37°C (A, D, E) or 30 min at 30°C (B, C). Mean control values ± SD of specific activities were: Succ.CoA-S: 0.030±0.002 (n=5), ALA-S: (2.530±0.708)$\times 10^{-3}$ (n=5), rhodanese: 102.2±8.4 (n=5), LDH: 910±82 (n=5), GLDH: 1,241±47 (n=5) where n = number of experimental determinations run in duplicate. Other experimental details were as described in Materials and Methods.

but under UV light it would proceed to remove PPy from the system and so decrease ALA-S activity.

At neutral pH UI is in its octacarboxylate form, so that binding to essential protonated lysine residues could occur thus reducing enzyme activity in the dark. An essential sulphydryl group is also located at the active center of ALA-S[31] so that additional light inactivation induced by UI could be attributed to photooxidation of this residue.

Rhodanese

Pure rhodanese is a labile enzyme which rapidly loses activity and for this reason all treatments were for only 30 minutes and at 30°C.

The enzyme activity decreased 40% in the dark and 70% under UV light. The presence of UI produced 82% and nearly complete (95%) inactivation in the dark and under UV light respectively (Figure 1C).

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We have found that preincubation of rhodanese for 30 minutes at 37°C in the absence of its substrates produced 56% inactivation, which was attributed to partial protein denaturation.[33]

It has been demonstrated that rhodanese contains four cysteine residues, one of which, Cys-247, is at the binding site of the sulphane sulphur atom of the substrate to yield the sulphur-substituted enzyme complex (E–S).[34] It has also been found that both the free enzyme and the E–S form were inactivated in the presence of reactive oxygen species due to oxidation of these essential sulphydryl groups with the generation of intramolecular sulphur bridges.[35] So, UI-induced dark inactivation of rhodanese is likely to be due to the binding of the porphyrin to thiol residues in the protein and the additional light inactivation due to photooxidation of Cys-247.

Lactate Dehydrogenase

LDH activity was unchanged when maintained for 2 hours at 37°C either in the dark or under UV light. In the presence of UI, activity was reduced 30% in the dark and 50% on illumination (Figure 1D).

It is well documented that LDH contains an essential histidine residue at its active site involved in the transfer of protons from and to its substrates.[36–38] This histidine residue is thus a very sensitive and likely target for UI attachment, so that modification by UI in the dark leads to LDH reduced activity. It is also known that photooxidation of this amino acid easily occurs by the action of porphyrins, explaining the 50% enzyme inactivation observed here in the presence of UI and UV light.

Glutamate Dehydrogenase

GLDH activity, in a similar manner to that of LDH, was not altered when maintained in the dark and under UV light. However, in the presence of UI, the enzyme activity was strongly reduced to nearly 80% irrespective of the light conditions used.

Because dark and light inactivation by UI was of the same magnitude, these effects can not be attributed to amino acid photooxidation.

An essential lysine at GLDH active site has been identified.[39] By analogy with ALA-S, at neutral pH UI can bind to lysine inducing structural changes in the protein, which could be responsible for enzyme inactivation observed both in the dark and light.

Finally, it has been shown that under these conditions, *in vitro* UI could induce light and dark inactivation of both cytosolic and mitochondrial enzymes. Studies on the change in content of total sulphydryl groups of these enzymes before and after their exposure to the porphyrin, and of other structural changes induced by UI are under examination to confirm the predictions made here.

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