JOURNAL OF PHYSICAL ORGANIC CHEMISTRY, VOL. 3, 69–76 (1990)

PORPHYRIN–TYROSINE INTERACTIONS: PHOTO-CIDNP AND NMR STUDIES

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Strong ^{1}H and ^{13}C nuclear polarizations are observed when aqueous solutions of synthetic water-soluble porphyrins are irradiated in the presence of tyrosine and some of its derivatives. These polarizations are strongly pH dependent. Evidence is also shown for the formation, in the dark, of a complex between the reactants. The association constants are evaluated from the NMR chemical shifts on the aromatic tyrosine protons induced by the presence of the porphyrins. The nature of the intermediate radical pair generating the CIDNP effects is discussed. An electron-transfer reaction from tyrosine to the excited triplet state of the porphyrin, or within the porphyrin–tyrosine excited complex, is expected to be the primary step in the reaction. It is followed by subsequent proton transfer within the initial ion radical pair. The spin polarizations arise principally from the back-transfer step, as the reactants are the only products which are polarized.

INTRODUCTION

The interactions between light, chemicals and living systems has generated a sudden burst of publications in recent years. Increasing interest has grown, for example, in the photochemical behaviour of drugs with photosensitizing properties. These properties can induce deleterious effects (phototoxicity, photoallergy, carcinogenicity, etc.) but the appropriate combination of light and chemicals has also resulted in therapeutic applications.[1] The mechanisms by which such a type of action is produced are generally poorly understood. They can be further complicated by the fact that the chromophore responsible for the photobiological effect may itself be a metabolite or a photoproduct. Hence a knowledge of the primary processes involved in these photoreactions is essential.

Photosensitized reactions are generally classified into two main groups: reactions due to radicals (type I) and reactions due to singlet oxygen (type II). Several physico-chemical techniques allow a deeper insight in that direction. Among them, photochemically induced dynamic nuclear polarization (photo-CIDNP) is well suited for type I reaction studies. It can give information about the short-lived radical intermediates and the reaction mechanisms. Thus the photochemical behaviour of the drug can be investigated in the absence[2] or presence[3,4] of some constituents of the biological substrates which are among their main targets in the living organisms: the pyrimidine and purine nucleobases (DNA, RNA) or the amino acids (proteins).

We report here the photo-CIDNP contribution to the photochemistry of porphyrins some derivatives of which are used in the treatment of solid tumours by porphyrin photodynamic therapy.[5] We studied their interaction with tyrosine as protein damage had been

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‡ Abbreviations: $TMPyPH_2$, *meso*-tetra(4-*N*-methylpyridyl)-porphyrin (chloride or tosylate); $TAPPH_2$, *meso*-tetra(4-*N*, *N'*, *N''*-trimethylaminophenyl)porphyrin (chloride); $TPPSH_2$, *meso*-tetra(4-sulphonatophenyl)porphyrin (sodium salt); *A*, enhanced absorption; CIDNP, chemically induced dynamic nuclear polarization; *E*, emission; ET, electron transfer; hfcc, hyperfine coupling constant; RPM, radical pair mechanism; PheOH, *N*-acetyltyrosine.

0894–3230/90/020069–08$05.00
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Received 12 December 1988
Revised 21 February 1989

established at the cellular membrane level.[6] Tyrosine was also the model previously chosen for testing the photoactivity of furocoumarin drugs.[4]

MATERIALS AND METHODS

The water-soluble porphyrins were synthesized according to published procedures.[7] *N*-Acetyltyrosine (PheOH) and the other tyrosine derivatives were purchased from Sigma (St. Louis, MO, USA). Deuterium oxide (99·8%) was from Spectrométrie Spin et Technique (Paris, France). All commercially available products were used without further purification.

Unless specified otherwise, the porphyrin and tyrosine concentrations were 2×10^{-3} M. The pH of the solutions was adjusted by adding small aliquots of DCl or NaOD (Spectrométrie Spin et Technique). It was measured directly inside the NMR tube. All values were uncorrected for the deuterium isotope effect.

The NMR spectra were run on a Bruker WP-80 spectrometer. The irradiation device has been described previously.[2] A CS 0·52 glass filter (Corning Glass Works, New York, USA) was added to cut off wavelengths under 330 nm, above which PheOH do not absorb the incident light.

^{1}H chemical shifts were calculated from 2,2-dimethyl-2-silapentane-5-sulphonate [DSS, δ(TMS) = 0·015 ppm]. The probe temperature was regulated at 318 K for routine experiments.

The ^{13}C NMR data (20·150 MHz) were obtained by utilizing FT/quadrature phase detection mode with a 10 mm probe; 2000 transients were required during light irradiation in 8K of memory with a pulse width of 3 μs (25° flip angle), a pulse delay of 1 s and broad-band proton decoupling. The 6024 Hz spectral width used resulting in an acquisition time of 0·68 s and a digital resolution of 1·47 Hz. Chemical shifts were from TMS by setting internal dioxane at δ = 67·86 ppm.

RESULTS

^{1}H CIDNP

When irradiated in aqueous solutions, porphyrins induce strong polarizations on *N*-acetyltyrosine. These polarizations depend on the nature of the porphyrin and on the pH of the solution. A typical CIDNP pattern of PheOH is shown in Figure 1 and has the following features: strong emission (*E*) on the $H_{3,5}$ aromatic ring protons (δ = 6·78 ppm; doublet) with a weak multiplet effect (AE) superimposed; enhanced absorption (*A*) on the β-CH_2 protons (δ = 2·75 ppm; multiplet); and a multiplet effect (*AE*) + a weak net effect (*A*?) on the $H_{2,6}$ aromatic ring protons (δ = 7·05 ppm; doublet).

Simultaneously, the cationic porphyrins, $TMPyPH_2$ and $TAPPH_2$, were also strongly polarized, as can be seen, for example, in Figures 1 and 2, respectively, for

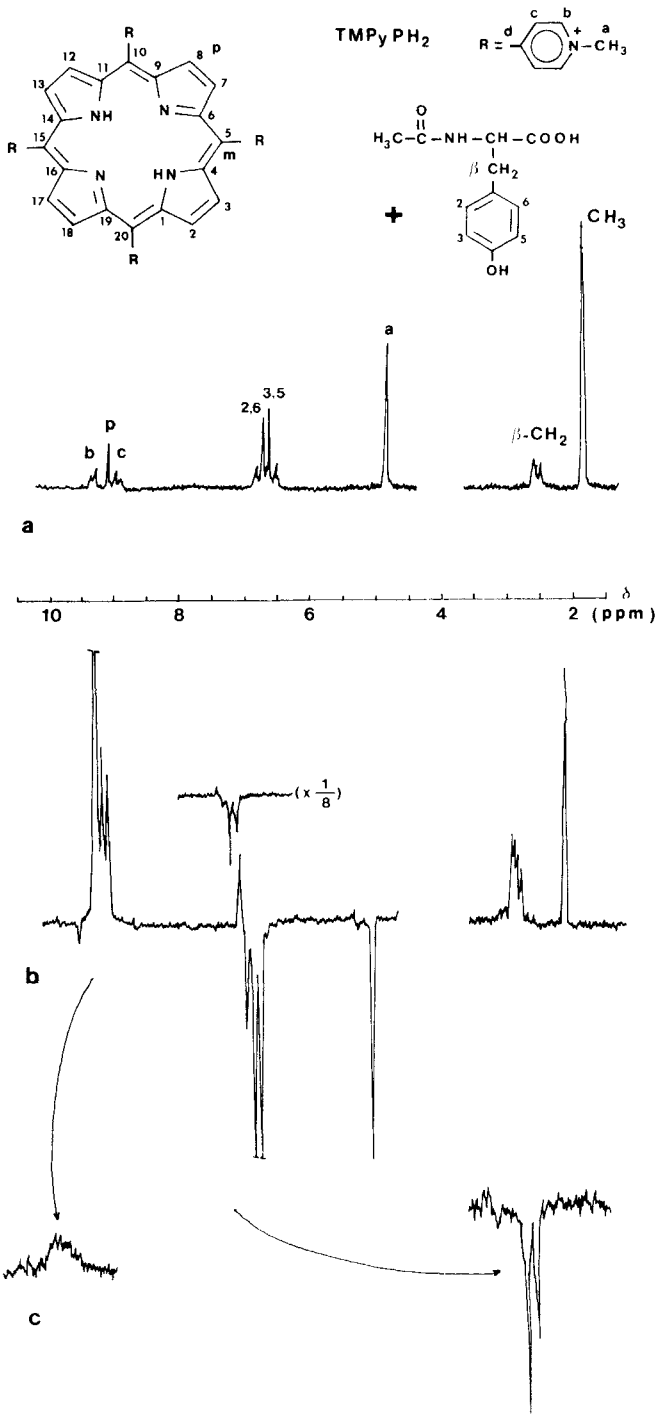

Figure 1. ^{1}H CIDNP spectrum: $TMPyPH_2$–PheOH–D_2O. (a) Before irradiation; (b) during irradiation, pH 4; (c) during irradiation, pH 12

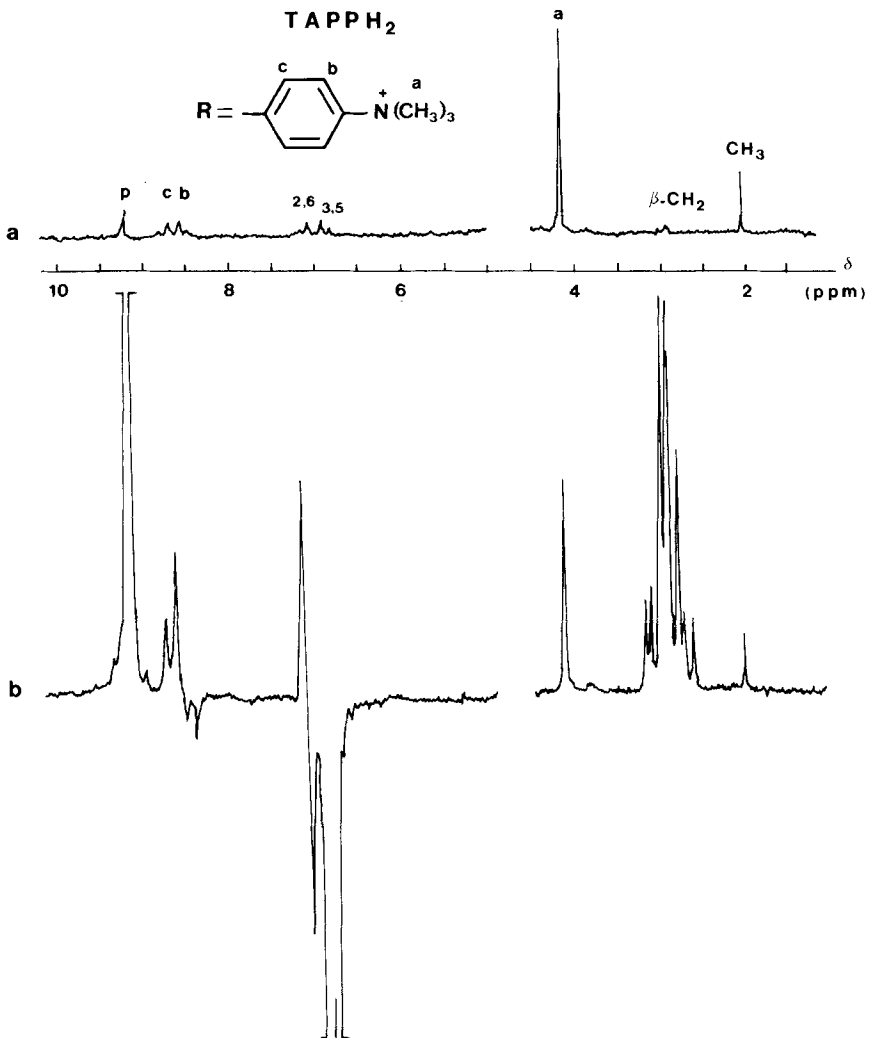

Figure 2. ^{1}H CIDNP spectrum: $TAPPH_2$–PheOH–D_2O at pH5. (a) Before irradiation; (b) during irradiation

each derivative: (i) for the porphine ring protons, *A* for the β-pyrroles, which is the largest polarization (protons p); (ii) for the *meso*-substituent protons, A and a multiplet effect which is difficult to characterize for the *meta*-aromatics (protons b), $E + AE$ effect for the *ortho*-aromatics (protons c) and *E* for the *N*-methyl protons of $TMPyPH_2$ (protons a, Figure 1). On the other hand, only line broadening was observed on the anionic porphyrin $TPPSH_2$.

Increasing the pH led to cancellation of the porphyrin polarizations (the signals were progressively broadened). Those on PheOH remained, but their intensities decreased accordingly. The same phenomena were still observed on the porphyrins when the latter were irradiated in aqueous solutions in the absence of any substrate or in the presence of guanine.[8]

^{13}C CIDNP

We also observed strongly polarized ^{13}C CIDNP spectra. The polarizations are reported in Table 1 for TMPyPH2.

Two important indications can be obtained from these spectra: the alternating polarizations on neighbouring carbon atoms of both the aromatic rings of PheOH and of the porphyrin pyridinium substituent indicate alternating hyperfine coupling constant (hfcc) signs in the radical intermediates from each reactant

Table 1. Spin polarizations of $TMPyPH_2$ and PheOH carbon atoms in aqueous solutions (pH 3)[a]

N-Acetyltyrosine			$TMPyPH_2$		
Atom	δ (ppm)	Polarization	Atom	δ (ppm)	Polarization
	—	—	C_m	116·97	*E*(strong)
C_1	128·79	*A*(weak)	C_d	158·79	*A*(strong)
$C_{2,6}$	130·76	*E*(weak)	C_c	130·76	*E*(weak)
$C_{3,5}$	115·88	*A*(strong)	C_b	145·27	*A*(weak)
C_4	155·08	*E*(weak)		—	—

[a]The different signals were attributed according to the published data by Surprenant *et al.*[32] for PheOH and by Goff and Morgan[33] for $TMPyPH_2$.

(alternant radical); and the strong polarizations on both carbon atoms which bind the substituent to the porphine ring, i.e. the *meso* atom (C_m) and the *para*-pyridine atom (C_d). They can be related to high values of the hfcc on these positions. Hence we can assume that the ring charge should be largely delocalized towards the pyridinium substituents.

Complexation phenomena

We noticed during our experiments that the PheOH aromatic pattern was considerably modified according to the ratio (ρ) of the PheOH concentration versus the porphyrin concentration. The chemical shifts (ca 6 and 7 ppm) of the $H_{3,5}$ and $H_{2,6}$ protons were affected by this ratio. This phenomenon is described in Figure 3 and may be ascribed to an association between both reactants. The corresponding CIDNP patterns are also shown under the NMR spectra in the dark. A complete study concerning the complexation between different amino acids and porphyrins will be published in detail elsewhere.[9] We report here the preliminary results obtained with our CIDNP experimental conditions.

The method described by Bouquant and Chuche[10] allows the description of the geometry of the complex. It is based on the analysis of the chemical shifts induced by the addition of porphyrin to aqueous solutions of PheOH. They are related to the reaction

$$xT + yP \rightleftharpoons T_xP_y \qquad (1)$$

where T represents PheOH, P the porphyrin and T_xP_y

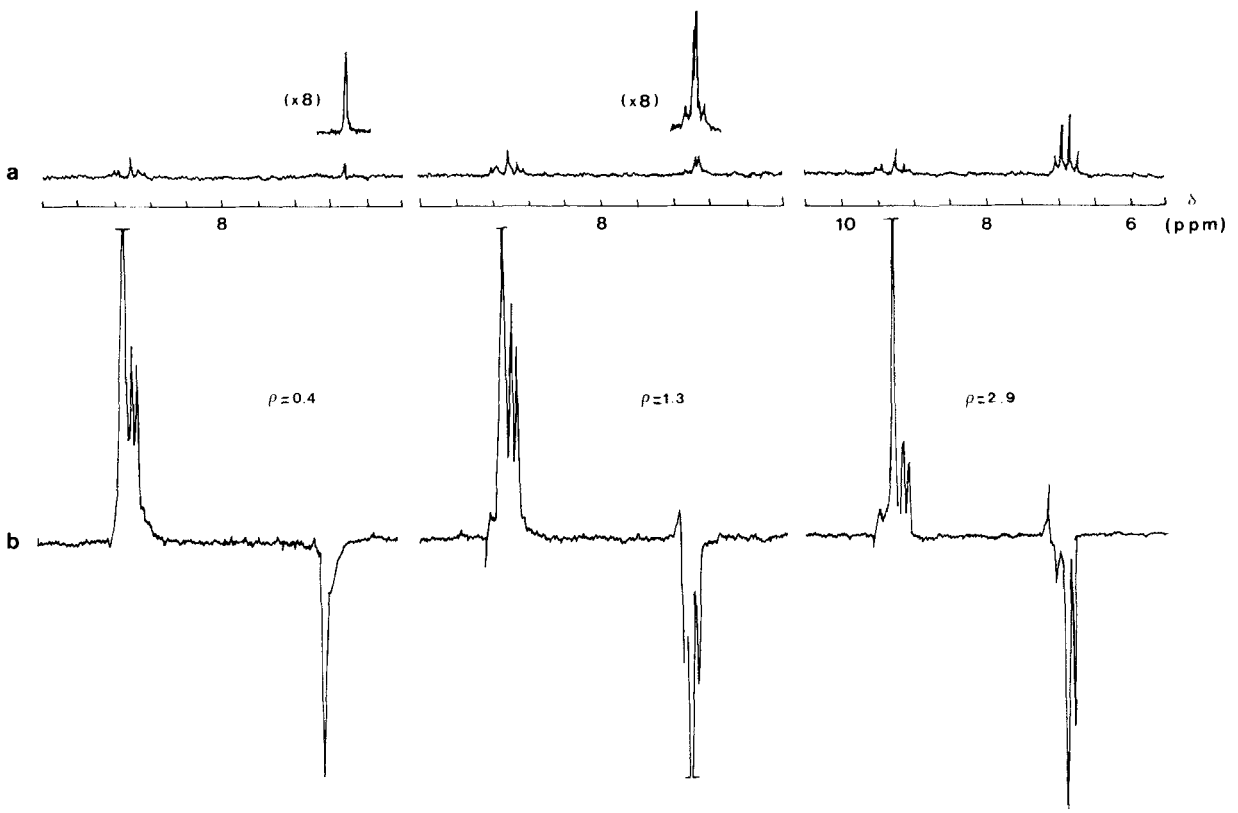

Figure 3. Influence of the concentration ratio (ρ) between PheOH and the porphyrin (here $TMPyPH_2$) (a) on the NMR spectrum in the dark and (b) on the CIDNP spectrum at 313 K (aromatic part of both reactants)

the complex. When the stoichiometry of the complex is assumed to be 1 : 1 ($x = y = 1$) and when considering equal initial concentrations of T and P ($T_0 = P_0 = C_0$), the following relationship could be written:

$$\Delta^i_T = -(\Delta^i_{TP}/K)^{1/2}(\Delta^i_T/C_0)^{1/2} + \Delta^i_{TP} \qquad (2)$$

where Δ^i_T is the induced chemical shift of the ith proton of T, Δ^i_{TP} is the chemical shift of the same proton in the complex TP and K is the equilibrium constant for formation of the complex. A plot of Δ^i_T vs $(\Delta^i_T/C_0)^{1/2}$ is then linear with a slope of $-(\Delta^i_{TP}/K)^{1/2}$ and an intercept of Δ^i_{TP}.

Such a typical plot is given in Figure 4 for the aromatic protons ($H_{2,3,5,6}$) of T at different temperatures. In the range of the studied C_0 concentrations (i.e. from $1{\cdot}2 \times 10^{-1}$ to $3{\cdot}3 \times 10^{-3}$ M), the pH of the solutions was between 4 and 5. The chemical shifts of the T protons were not affected in this range of pH values when T was dissolved in solution in the absence of the porphyrin.

The Δ^i_{TP} and K values and the corresponding correlation coefficients (r) are given in Table 2 as an example for the PheOH aromatic protons. We did not take into account the activity coefficients in these calculations. The errors are also minimized since the saturation factor $s = \Delta^i_T/\Delta^i_{TP}$ lies in the range 0·2–0·8, where the method proves to be suitable.[10]

From Table 2 it can be seen that the K values regularly decreased when the temperature was increased whereas Δ^i_{TP} are not affected. This suggests that the complex geometry is independent of temperature.

From a plot of log K vs T^{-1} we determine that for the T–P association $\Delta H^0 = -26{\cdot}7 \pm 0{\cdot}4$ kJ mol^{-1} and

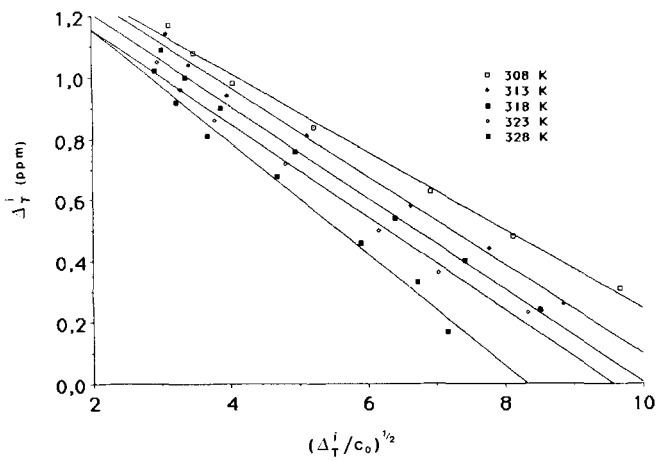

Figure 4. Induced chemical shift (Δ^i_T) of PheOH protons versus $(\Delta^i_T/C_0)^{1/2}$ at different temperatures; $i = H_{2,3,5,6}$

Table 2. Typical constants obtained from the plot of Δ^i_T vs $(\Delta^i_T/C_0)^{1/2}$ in the PheOH–TMPyPH$_2$ complex at different temperatures; $i = H_{2,3,5,6}$

T (K)	r	K (l mol^{-1})	Δ^i_{TP} (ppm)
308	−0·9970	92·9 ± 7·7	1·53 ± 0·40
313	−0·9975	73·7 ± 7·5	1·55 ± 0·50
318	−0·9980	66·0 ± 5·4	1·52 ± 0·40
323	−0·9957	62·5 ± 8·4	1·46 ± 0·60
328	−0·9945	45·5 ± 4·8	1·52 ± 0·60

$\Delta S^0 = -49{\cdot}1 \pm 1{\cdot}2$ J mol^{-1} K^{-1} as we have the relationship:

$$\log K = -\Delta H^0/RT + \Delta S^0/R \qquad (3)$$

Other PheOH derivatives

Similar results were obtained when *N*-acetyltyrosine was replaced with tyrosine ethyl ester or by L-Dopa.

On the other hand, CIDNP effects were no longer available with 4-methoxyphenylalanine (PheOMe) or phenylalanine (Phe). For the temperature and pH conditions under which the CIDNP experiments were performed, no evidence was found for association of the porphyrins with these last two derivatives. Nevertheless, it was established that, at room temperature and in alkaline media, complexes were also formed with Phe, although the equilibrium constants were half those measured with PheOH under the same conditions.[9]

DISCUSSION

Complexation phenomena

Porphyrins are known to complex in the ground state with several electron-donating agents.[11,12] The type of interaction depends on the nature of both interacting partners; 1 : 1 or 1 : 2 molecular complexes can be formed.[13] Pasternack *et al.*[14] reported $\pi-\pi$ complexes between monomeric cationic porphyrins and nucleic acids. On the other hand, Heathcote *et al.*[15] have shown that haematoporphyrin and tryptophan interacted through hydrogen bond formation.

The photochemical behaviour of such systems interacting in the ground state should be drastically influenced by the nature of the complex. A strong interaction could prevent any photoreactions.[16] On the other hand, a moderate association putting the two partners in close proximity to each other can favour charge-transfer reactions. These considerations should be of great interest and play a comprehensive role in the study of the interactions between porphyrins and

proteins in biological systems. Hence, as an example, the porphyrins are in contact with proteins in the cells and they will preferentially interact with the aromatic residues, e.g. tyrosine and tryptophan.

We have found low values of the stability constants (e.g. $K = 66{\cdot}0 \pm 5{\cdot}4\,l\,mol^{-1}$ for the aromatic protons of PheOH at 318 K). Moreover, the β-CH_2 proton chemical shift [Δ^i_{TP} (mean value) = $2{\cdot}22 \pm 0{\cdot}60$ ppm] is influenced more by the complexation than the aromatic protons [Δ^i_{TP} (mean value) = $1{\cdot}52 \pm 0{\cdot}50$ ppm]. This indicates that the former group is included in the complexation site. For each PheOH proton we also calculated a correlation coefficient close to 1 between Δ^i_T and $(\Delta^i_T/C_0)^{1/2}$ (see Table 2 as an example for the aromatic protons). Hence the complex stoichiometry should be 1:1, as assumed in the calculations. The complexation therefore probably involves an overlap of the π-systems of each species, with a moderate association. The geometry of the complex and the ring-current effect by the porphyrin ring will then cause an upfield shift of the tyrosine aromatic protons.

Similar results were obtained for $TAPPH_2$ and $TPPSH_2$, but we did not make systematic calculations with these compounds. They are more critical to interpret in the case of $TPPSH_2$, which is likely to be non-monomeric in the solutions that we used.

CIDNP effects

The analysis of the PheOH CIDNP pattern indicates that, in the intermediate radical species from which the polarizations are originated, the highest hfccs will be located on $H_{3,5}$ and β-CH_2 and they will be of opposite signs. The $H_{2,6}$ hfcc will be far smaller than and have the same sign as the β-CH_2 hfcc. This could agree with the spin distribution of both a neutral $PheO^{\cdot}$ radical or a cationic $PheOH^{\cdot+}$ radical. The magnetic parameters for $PheO^{\cdot}$ are well known:[17] $g = 2{\cdot}0046$; $a_{H_{3,5}} = -6{\cdot}15$ G; $a_{H_{2,6}} = +1{\cdot}5$ G and $a_{\beta\text{-}CH_2} = +7{\cdot}7$ G. The cation radical was not described, but in analogy with *p*-cresol[18] its parameters could be estimated as $g = 2{\cdot}0031$; $a_{H_{3,5}} = -4.5$ G and $a_{H_{2,6}} = +0{\cdot}05$ G.

From a mechanistic point of view, generally two types of reactions are observed when derivatives possessing a phenol group are irradiated in the presence of photosensitizers:[19-21] electron transfer [ET, equation (4)] or direct hydrogen abstraction [equation (5)]. Such reactions should lead in our case to the following radical pairs according to the primary steps occurring:

$$^*PH_2 + PheOH \rightarrow \overline{PH_2^{\cdot-}\,PheOH^{\cdot+}} \qquad \textit{Pair I} \quad (4)$$

$$^*PH_2 + PheOH \rightarrow \overline{PH_3^{\cdot}PheO^{\cdot}} \qquad \textit{Pair II} \quad (5)$$

where PH_2 represents the porphyrin derivative. The PheOH cation radical can easily deprotonate as pK ($PheOH^{\cdot+}/PheO^{\cdot}$) is negative.[22] Hence from pair I the following pairs can also be formed:

$$\overline{PH_2^{\cdot-}\,PheOH^{\cdot+}} \rightarrow \overline{PH_2^{\cdot-}\,PheO^{\cdot}} + H^+ \qquad \textit{Pair III} \quad (6)$$

$$\text{or } \overline{PH_2^{\cdot-}\,PheOH^{\cdot+}} \rightarrow \overline{PH_3^{\cdot}PheO^{-}} \qquad \textit{Pair II} \quad (7)$$

Reaction (7) will be favoured in slightly basic (pH < 9), neutral or acidic media, as Neta *et al.*[23] have shown that the radical derived from the tetracarboxy derivative of tetraphenylporphin is mostly in the anionic form at pH > 9·7.

Now, as polarizations on PheOH are also observed in media for which the pH is higher than 11, where PheOH is in its anionic form [p*K* (PheOH/$PheO^-$) = 10·4],[24] the hydrogen abstraction reaction is no longer available and ET will then be the only feasible process according to the equation:

$$^*PH_2 + PheO^- \rightarrow \overline{PH_2^{\cdot-}\,PheO^{\cdot}} \qquad \textit{Pair III} \quad (8)$$

Consequently, three pairs can be formed from such charge-transfer interactions between the porphyrin and the amino acid. According to the cyclic scheme depicted by Hore and Kaptein,[20] the spin polarization from each pair should arise from the reverse step leading back to the reactants, as no signals other than those from the reactants are observed on the spectra. In such types of reactions CIDNP is preferentially detected from the geminate recombination as the opposite spin polarization of the escape pathway is attenuated by relaxation during the lifetime of the escaping radicals.

Let us now consider the multiplet effect observed on the $H_{2,6}$ protons. This effect is invariant when the spectrum is recorded with a flip angle of 90° or when it has a lower value. According to Schäublin *et al.*,[25] the multiplet effect should collapse for 90° flip angles. However, Boelens *et al.*[26] reported multiplet effects even for such values. They attributed this phenomenon to magnetic field inhomogeneity.

The application of the second Kaptein rule[27] to this multiplet effect observed on the $H_{2,6}$ protons of PheOH (*AE* effect, $\Gamma m_{H_{2,6}} < 0$) allows the determination of the spin multiplicity of the correlated radical pair. All the parameters will be the same for each pair: I, II or III as the hfccs ($a_{H_{2,6}}$ and $a_{H_{3,5}}$) have the same signs in the anion or neutral radical of PheOH. Thus, cage recombination ($\varepsilon > 0$), a positive nuclear spin–spin coupling ($J_{H_{2,6}\text{-}H_{3,5}} > 0$), and a positive position parameter ($\sigma > 0$, the nuclei are on the same radical) lead to a triplet precursor ($\mu > 0$).

Now, the net effect on the $H_{2,6}$ protons is more difficult to assign from the ^1H CIDNP spectrum (see Figure 1 or 2), but it can be deduced from the carbon atom polarizations (Table 1). As generally the hfcc signs are opposite for the carbon atom and the hydrogen atom directly attached to it, the net effect will thus be opposite for each atom. *E* is observed for $C_{2,6}$, hence the net effect for $H_{2,6}$ should be *A* ($\Gamma n_{H_{2,6}} > 0$). The signs of the parameters μ, ε and $a_{H_{2,6}}$ remain the same as those used in the multiplet effect (see above), and the

first Kaptein rule[27] leads to a g-factor difference which should be positive [$\Delta g = g(\text{PheO}^{\cdot} = 2{\cdot}0046$ or $\text{PheOH}^{\cdot+} = 2{\cdot}0031) - g(\text{porphyrin-derived radical}) > 0$]. This is established for $PH_2^{\cdot-}$ ($g \approx 2{\cdot}0027$) and should be very likely[8] for $PH_3^{\cdot}$ with both PheOH-derived radicals. In the case of the neutral radical $\text{PheO}^{\cdot}$ (pair II or III), Δg will be larger than for the cation radical $\text{PheOH}^{\cdot+}$ (pair I). The net effect should then dominate according to the RPM theory of CIDNP.[28] It is really the case for the more strongly coupled protons $H_{3,5}$. Their interaction with the more weakly coupled protons $H_{2,6}$ can explain the multiplet effect observed on the latter, despite a large g-factor difference in the radical pair.

As could be seen from equations (5) and (7), ET and direct hydrogen abstraction will give the same neutral radical pair (pair II). Moreover, the photochemical hydrogen abstraction reaction takes place, frequently subsequent to the mechanism of charge transfer followed by proton transfer.[29] This may also be the case here as the protonation of the anion radical $PH_2^{\cdot-}$ is expected to be easier than that of the parent molecule.[23]

The absence of polarizations on PheOCH_3 is related to the fact that neither ET[4] nor hydrogen abstraction is feasible with this derivative. Further no evidence was found for association of the latter with the porphyrins under our experimental conditions.

On the other hand, the free-energy change in the formation of pair I [equation (4)] should become endergonic[8] in acidic media (pH < 4·8) as the one-electron oxidation potential of PheOH increases from basic to acidic media.[30] The spin polarizations on PheOH are very strong in such media, however. Hence the charge transfer will then occur within the porphyrin–tyrosine complex, confirming that a moderate association should enhance the photochemical reactivity of the system.

These different considerations lead us to propose that pair II should be the central intermediate when the pH does not exceed 10·4. It can be formed according to ET from PheOH to a porphyrin excited triplet state [equation (4)], or within a porphyrin–tyrosine complex (see above) followed by proton transfer within the initial correlated ion radical pair I [equation (7)]. Nevertheless, we cannot completely exclude hydrogen atom transfer [equation (5)] which leads to the same radical pair. The spin polarizations will then arise from the back-transfer steps:

$$\overline{^3PH_3^{\cdot}\,\text{PheO}^{\cdot}} \rightarrow PH_2^{\dagger} + \text{PheOH}^{\dagger} \qquad (9)$$

$$\overline{^3PH_3^{\cdot}\,\text{PheO}^{\cdot}} \rightarrow PH_3^{\cdot} + \text{PheO}^{\cdot} \qquad (10)$$

$$PH_3^{\cdot} + \text{PheO}^{\cdot} \rightarrow PH_2^{\dagger} + \text{PheOH}^{\dagger} \qquad (11)$$

where the recombination step [equation (9)] will be preponderant over the escape pathway [equations (10) and (11)] in the CIDNP effects (see above). A dagger (†) denotes nuclear spin polarization.

For pH > 10.4, neither proton transfer nor hydrogen abstraction is feasible. Hence ET should be the only process accounting for the observed polarizations. Pair III is formed in this case [equation (8)]. Reverse ET within the latter will then lead to $PH_2^{\dagger}$ and $\text{PheO}^{-\dagger}$. The disappearance of CIDNP due to PH_2 in basic media could thus be attributed to extensive electron exchange broadening[31] between $PH_2^{\cdot-}$ and the parent molecule.

Further the progressive decrease in the polarizations on PheOH, which is observed with increasing pH, can be related to less efficient proton or hydrogen atom transfer when the solution becomes more alkaline. Nevertheless, CIDNP effects on PheOH never totally collapse, even in very basic media. This confirms that in slightly basic media (pH < 10·4) CIDNP does not proceed exclusively through an ET mechanism. The latter should be followed by proton transfer or hydrogen abstraction should occur simultaneously, both processes leading to an increase in the polarizations.

In conclusion, the pH drastically influences the charge-transfer reaction between tyrosine and the porphyrins. Further, the aggregation phenomena or the possibility of complexation with some substrates make the study of such interacting systems very critical. Hence the latter could not be used as a model for establishing a simple test to study the photoactivity index of the different porphyrins as too many parameters remain to be controlled.

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