Structure of the Y_D Tyrosine Radical in Photosystem II. Determination of the Orientation of the Phenoxyl Ring by Enantioselective Deuteration of the Methylene Group

S. A. M. Nieuwenhuis,† R. J. Hulsebosch,‡ J. Raap,† P. Gast,‡ J. Lugtenburg,† and A. J. Hoff*,‡

Leiden Institute of Chemistry, Gorlaeus Laboratories
Department of Biophysics, Huygens Laboratory
Leiden University, P.O. Box 9504
2300 RA Leiden, The Netherlands

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In the reaction center of photosystem II (PS II), two tyrosyl residues, D1 Tyr161 (Y_Z) and D2 Tyr160 (Y_D) (*Synechocystis* notation), apparently symmetrically positioned with respect to the primary electron donor,[1] are redox-active, but their redox properties are quite different. For example, Y_Z mediates fast electron transfer from the Mn cluster of the oxygen-evolving complex (OEC) to the photo-oxidized primary electron donor P680$^+$, while Y_D is a much slower electron donor to P680$^+$ and is not involved in primary electron transport. Both $Y_Z^•$ and $Y_D^•$ are neutral, unprotonated radicals; $Y_Z^•$ is unstable, and $Y_D^•$ can be kept for many hours. The considerable difference in redox properties of Y_Z and Y_D, and generally of tyrosyl electron transport components in a number of enzymes, must be related to different interactions with the protein matrix or protein-induced structural differences or both. For example, a difference in hydrogen bonding to the phenoxyl oxygens has been implicated, $Y_D^•$ showing a well-ordered H-bond and $Y_Z^•$ showing a highly disordered one.[2] Furthermore, the configuration of the β-methylene hydrogens shows a much broader distribution for $Y_Z^•$ than for $Y_D^•$, suggesting a more flexible phenoxyl ring for $Y_Z^•$.[3] The observed differences in radical properties have suggested models for oxygen evolution in which Y_Z acts as an abstractor of hydrogens or protons from water bound to the Mn cluster of the OEC.[4] One important structural parameter reflecting interactions between the tyrosyl redidues and the protein matrix is the orientation of the phenoxyl ring with respect to the polypeptide backbone. This orientation can be experimentally assessed through the hyperfine interactions (hfi) of the β-methylene hydrogens, which have been shown to be different both in reaction centers of PS II[5] and in the tyrosyl radical of ribonucleotide reductase.[6] Two possible orientations of the aromatic ring result, as shown in Figure 1. Until now, it has not been possible to discriminate between these orientations. We have, via enantioselective deuteration of each of the two β-hydrogens, incorporation in PS II of a kanamycin resistant mutant of *Synechocystis* sp. PCC 6803,[7] and EPR measurements, determined that in $Y_D^•$ the prochiral H_R has the largest hfi, corresponding with the small dihedral angle (conformation A in Figure 1). In vitro, however, the situation is reversed, as H_S has the largest hfi, corresponding to conformation B in Figure 1. It follows that the phenoxyl ring of $Y_D^•$ is highly constrained compared to the in vitro equilibrium configuration, explaining the small width of the orientational distribution of its β-methylene hydrogens earlier observed.[3a]

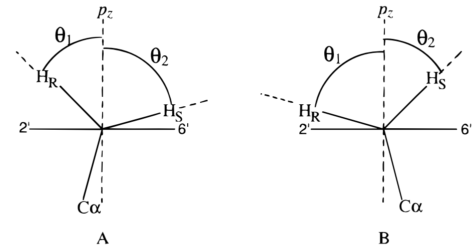

Figure 1. Two possible orientations of the phenoxyl ring relative to the methylene group. The prochiral β-methylene hydrogens are indicated as H_R and H_S. The plane of the aromatic ring (horizontal line) is perpendicular to the plane of the paper; the view is down the Cβ−C1′ bond. θ_1 and θ_2 represent the dihedral angles between the 2p_z orbital at C1′ and the planes containing the C1′−Cβ−H_R bonds and the C1′−Cβ−H_S bonds, respectively.

The isotropic β-methylene hydrogen hfi A_i^{iso} is, via hyperconjugation, directly related to the orientation of the β-hydrogen with respect to the plane of the tyrosine phenoxyl ring and the spin density at the nearest ring carbon C1′ by the simplified expression[8] $A_i^{iso} = 5.8\rho_{C1'} \cos^2 \theta_I$ [mT].

Small alterations of the so-called dihedral angles θ_i ($i = 1, 2$ and $\theta_1 = 120° - \theta_2$; ideal sp^3 hybridization) between the C1′−Cβ−$H_{\beta j}$ plane (j = *R*,*S*[9]) and the p_z-orbital of C1′, which are species dependent, drastically influence the magnitude of the isotropic hfi and therefore basically determine the structure of its EPR spectrum. This influence does not arise from variations in the unpaired electron spin density at the tyrosine ring carbon C1′ ($\rho_{C1'}$), since the unpaired spin density at this position is relatively constant for the presently known tyrosyl radicals ($0.32 < \rho_{C1'} < 0.38$).[10] Generally, both β-hydrogen hfi are inequivalent due to different dihedral angles. For tyrosyl radicals in vitro (in frozen alkaline solution), dihedral angles of 75° and 45° have been suggested,[3b,c] with a homogeneous distribution of 30° attributed to a rotational mobility about the C1′−Cβ bond prior to freezing. For tyrosine radicals in vivo, for instance for $Y_D^•$ of PS II or for the tyrosyl radical in *Escherichia coli* ribonucleotide reductase, dihedral angles of 52° and 68° (with a small conformational distribution of about 4°)[3a] and 13° and 90°[6b] were found, respectively. These observations strongly suggest that the conformation of the tyrosyl methylene groups is controlled by the local protein environment. To unequivocally determine the ring orientation, we have applied site-selective deuteration of the β-methylene site. Exchanging a hydrogen to a deuterium nucleus reduces the hfi by a factor of about 6.5. Site-selective deuteration of one of the β-hydrogens will therefore provide us information

† Leiden Institute of Chemistry.
† Department of Biophysics.

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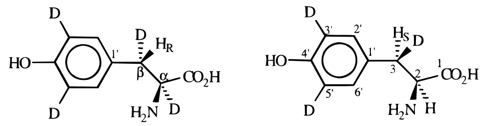

1a **1b**

Figure 2. Structure of 2(*S*),3(*S*))-[2,3,3′,5′−D_4]tyrosine (**1a**) and 2(*S*),3-(*R*))-[3,3′,5′−D_3]-tyrosine (**1b**). Because of the negligible spin density at the α-position in the tyrosine radical, deuteration at this position in tyrosine **1a** does not influence the spectra.

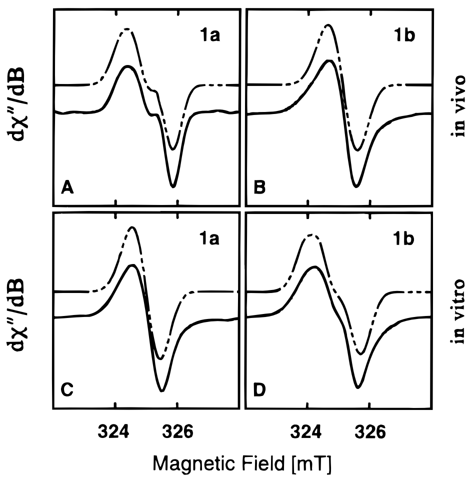

Figure 3. Experimental (solid line) and simulated (dashed line) X-band (9.089 GHz) EPR spectra of (top line) *Synechocystis pDICK* whole cells with incorporated enantioselectively deuterated tyrosine **1a** (A) and **1b** (B), (bottom line) in vitro radicals of tyrosine **1a** (C) and **1b** (D). Experimental conditions: modulation amplitude 0.25 mT (in vivo) and 0.025 mT (in vitro); scan speed 2.5 mT/min; time constant 1.0 s; microwave power 50 μW; temperature 90 K.

about the magnitude of the hfi of the remaining (unchanged) hydrogen, since the EPR spectrum will be dominated by the hfi of this hydrogen (provided the 3′,5′ positions are also deuterated).

Synthesis of the enantioselective β-methylene deuterated tyrosines was carried out as described.[11] The structure of both synthesized tyrosines are shown in Figure 2. Both hydrogens at the 3′ and 5′ positions were also exchanged by deuterium, to enhance the effect of the β-hydrogen labeling. The labeled tyrosines were incorporated in *Synechocystis pDICK* according to the method of Barry and Babcock.[12] Cultures were grown to late log phase, after which the cells were pelleted by centrifugation, resuspended in glycerol, and inserted into a quartz EPR tube. Prior to storage in liquid nitrogen, the sample was illuminated with white light at room temperature for 1 min followed by 2 min of dark adaptation. In vitro tyrosine radicals were generated by 2 min of UV illumination (250−350 nm) of tyrosine dissolved (5 mM) in a deoxygenated NaOD/D_2O pH 11 (meter reading) solution at 77 K. EPR spectra were recorded with a Varian E-9 X-band (9.2 GHz) spectrometer equipped with a nitrogen gas flow cryostat. The spectra were recorded at 90 K and were simulated using a powder EPR simulation program.[13] Six independent cultures were grown (three with **1a**, three with **1b**), and three independent in vitro samples were prepared with each labeled tyrosine; typical EPR spectra are shown in Figure 3. After the EPR spectra were recorded, the incorporation of the labeled tyrosines in the samples was measured as follows. The cells were broken in a Bead-Beater, and the membrane fragments were isolated by centrifugation and thoroughly washed with water. Hydrolysis was effected as published;[14] derivatization of the resulting free amino acids was performed according to Hušek.[15] Incorporation was determined with GC-MS (GC Chrompack 25 m fused silica column, CP-sil-5CB 0.25 mm i.d.; MS ITD 700, Finnigan MAT). Incorporation of **1a** and **1b** in the cells was 72% and 60%, respectively.

Table 1. Assignment of the Dihedral Angles θ_1 and θ_2 (see Figure 1) in Y_D• of *Synechocystis PDICK* (in vivo) and in the Tyrosyl Radical (in vitro)

	θ_1 (deg)	θ2 (deg)
in vivo	52 (±4)	68 (±4)
in vitro	75 (±15)	45 (±15)

We have neglected the small mass dependence of hyperconjugation due to zero-point energy effects, because in the literature no isotope effect on the methylene group hfi is reported. Note that the dihedral angle is independent of changes in the Cβ−H bond length.

The top two traces in Figure 3 display the X-band EPR spectra of the specifically deuterated tyrosine Y_D radicals in *Synechocystis pDICK* cells. A distinct splitting in Figure 3A indicates that the H_R hydrogen (see Figure 2, compound **1a**) gives rise to the major hydrogen hyperfine interaction corresponding to the 52° dihedral angle. On the other hand, deuteration of this H_R hydrogen (namely, in tyrosine **1b**) will leave only the much smaller second H_S hydrogen hfi contributing to the spectal line shape. This is reflected in Figure 3B where almost no hfi is observed. The parameters used for simulating these spectra were taken from Tommos et al.;[16] both experimental spectra were corrected for the 28/40% nonlabeled tyrosines in the cells. Comparison with the in vitro radicals in frozen solution reveals remarkable changes. Here, the major hfi originate from the H_S hydrogen instead of the H_R hydrogen (Figure 3C,D). Simulation of the in vitro radical spectra was carried out with the parameter set reported by Hulsebosch *et al.*[13] and included a static (rectangular) conformational distribution of the β-hydrogens ($\theta_1 = 75 \pm 15°$ and $\theta_2 = 45 \pm 15°$). These values for θ_1 and θ_2 correspond well with those found for tyrosine crystals ($\theta_1 = 72-73°$ and $\theta_2 = 47-48°$).[17]

The results summarized in Table 1 clearly show that the conformation of the aromatic ring of $Y_D^\bullet$ with respect to the polypeptide backbone (Figure 1A) is different from that in vitro (Figure 1B) and is highly constrained due to interactions with the local protein environment. Our unambiguous assignment of the hydrogen hfi of the prochiral β-methylene hydrogens H_R and H_S provides conclusive evidence that $Y_D^\bullet$ is strongly immobilized, locking the phenoxyl ring in one particular position, thereby ensuring a firm hydrogen bond of the phenoxyl oxygen to the protein matrix. Likely, the observed differences in H-bonding to the phenoxyl oxygens of $Y_Z^\bullet$ and $Y_D^\bullet$ [2] are related to a difference in equilibrium position of the phenoxyl ring. Work is currently in progress to determine the H_R and H_S hydrogen hyperfine interactions in $Y_Z^\bullet$.

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