Binding of Nitric Oxide to Reduced L-Tryptophan-2,3-dioxygenase as Studied by Electron Paramagnetic Resonance*

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Ferrous L-tryptophan-2,3-dioxygenase reacts with nitric oxide both in the presence and in the absence of L-tryptophan. Electron paramagnetic resonance studies suggest that the proximal ligand of the heme is a nitrogen atom, probably from an histidyl residue. The interaction of the protein with substrate changes both the symmetry of the paramagnetic center and the mode of interaction of the iron atom with its two axial ligands, NO and the proximal nitrogen atom. Optical absorption and EPR spectra suggest that the affinity of NO for tryptophan dioxygenase increases in the order: tryptophan dioxygenase, tryptophan dioxygenase + α-methyltryptophan, tryptophan dioxygenase + 5-hydroxytryptophan, tryptophan dioxygenase + L-tryptophan. A possible correlation between the number of superhyperfine lines in the EPR spectrum and the affinity of the enzyme for NO is discussed.

The interaction of molecular oxygen with hemoproteins leads to a structural intermediate which is subsequently broken down according to the function of the protein molecule. While hemoglobin carries oxygen by reversible binding and cytochrome c oxidase reduces it to water, L-tryptophan-2,3-dioxygenase catalyzes the insertion of 2 atoms of the molecular oxygen into the pyrrole ring of L-tryptophan to yield N-formylkynurenine. The enzyme extracted from a Pseudomonad is a tetramer containing 2 hemes/molecular weight of 120,000 (1). In the presence of L-tryptophan, the reduced enzyme forms an intermediate in the presence of oxygen with optical absorption bands at 418 nm, 545 nm, and 580 nm, bearing strong resemblance to those of oxyhemoglobin (2, 3). However, in the absence of substrate, no such intermediate has been observed. Carbon monoxide is a competitive inhibitor of the enzyme activity with respect to O2 binding. The affinity of CO for the ferrous enzyme is much greater in the presence of L-tryptophan than in its absence (2, 4, 5), and thus, the ferrous protein can exist in a form with either low affinity or high affinity for exogenous ligands dependent upon the presence of a structural modifier, in this case, the substrate. Likewise, the affinity of the ferric protein for exogenous ligands such as cyanide is mediated in similar fashion by the presence of substrate (2, 6). The enzymatic activity is regulated by a homotropic interaction (1). In fact, the plots of reaction velocity versus L-tryptophan concentration manifest a sigmoidal curve, indicating that multiple binding sites for L-tryptophan are involved in the reaction. The addition of α-methyltryptophan, which is neither a substrate nor an inhibitor normalizes the sigmoidal relationship and hence α-methyltryptophan is believed to be bound to a regulatory site distinct from the catalytic site. On the other hand, 5-hydroxytryptophan is a competitive inhibitor with respect to L-tryptophan and hence is believed to be bound at the catalytic site (1).

In view of a better understanding of the mechanism of the enzymatic activity of tryptophan dioxygenase and of its regulation and assuming a close analogy between nitric oxide and oxygen in their binding to heme, we have undertaken an EPR study of the fixation of NO to this protein. Since NO has one free electron, nitrosyl ferrous hemoproteins are odd spin paramagnetic species (S = 1/2) and are amenable for study with this technique. In many instances, EPR has led to structural information concerning the nature of the heme proximal ligand (7), the symmetry of the paramagnetic nitrosyl-heme complex (8, 10) and the nature of the interactions between the heme sites and the effector sites (10-12).

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1 The abbreviations used are: tryptophan dioxygenase and tryptophan dioxygenase-NO, L-tryptophan-2,3-dioxygenase and its ferrous nitrosyl derivative; DPPH, 1,1-diphenyl-2-picrylhydrazyl stable radical.
EPR Study of Nitrosyl Tryptophan-2,3-dioxygenase

MATERIALS AND METHODS

L-Tryptophan-2,3-dioxygenase was prepared from Pseudomonas fluorescens (ATCC 11299) as previously described (3). Ferrous nitrosyl derivatives were prepared in a Thunberg tube, provided with a 1-cm optical cell and a side arm. To the ferric enzyme (heme concentration from 35 to 100 μM) in Tris-acetate buffer (50 mM, pH 7.0) under an argon atmosphere were added sodium nitrite to a final concentration of 10 mM and a small amount of sodium dithionite. The nitric oxide compound formed was transferred anaerobically to an EPR tube and immediately frozen in liquid nitrogen. In some instances, the enzyme was reduced in the Thunberg tube with dithionite and then reacted with nitric oxide at 1 atm for 10 min. Substrate and effectors were added from a side arm of the Thunberg tube. EPR spectra, taken at 77 K, were recorded with a Varian E-9 spectrometer operating at 9.16 GHz with a 100 KHz magnetic field modulation of 2 G. The microwave power was 50 milliwatts. DPPH in benzene was used as a field marker. EPR signal intensities were obtained by manual integration.

RESULTS

Optical Spectra—In the presence of L-tryptophan, tryptophan dioxygenase-NO has an absorption spectrum with broad bands at 418, 540, and 568 nm, typical of ferrous nitrosyl hemoproteins (Fig. 1). However, in the absence of substrate the optical spectrum is more complex, with a shoulder near 585 nm and an absorption maximum at 552 nm. The Soret absorption is broad and composite, showing a maximum at 430 nm and a shoulder near 418 nm. We interpret the spectrum obtained in the absence of L-tryptophan as reflecting the superposition of the optical spectrum of the dioxygenated ferrous enzyme with absorption maxima at 432, 554, and 588 nm (3) and that of the nitrosyl enzyme described above. In this interpretation we suppose that L-tryptophan increases the affinity of the enzyme for NO (see "Discussion").

EPR Spectra—The EPR spectra of tryptophan dioxygenase-NO in frozen solution at 77 K were found to be extremely dependent on the presence of substrate, effector, or inhibitor. The EPR spectrum obtained with the native enzyme in the absence of any substrate or effector is typically that of a reduced nitrosyl-heme compound (9), indicative of rhombic symmetry at the paramagnetic center (Fig. 2A, Spectrum 1; and Table I). A well resolved superhyperfine pattern, consisting of essentially three lines, separated by 16 G and 17.5 G is observed at $g_2 = 2.009$ (Fig. 2B, Spectrum 1). The addition of substrate, L-tryptophan, modifies the EPR spectrum of tryptophan dioxygenase-NO in several ways. First, the symmetry is changed, reflected in the alteration of EPR line shape (Fig. 2A, Spectrum 2; and Table I). Second, the superhyperfine structure centered at $g_2 = 2.004$ is composed of nine very regularly spaced lines that can be grouped as a triplet (coupling 20 G) of a triplet (coupling 7.0 G) (Fig. 2B, Spectrum 2). In comparison, the addition of the effector, α-methyltryptophan, the inhibitor, modified the EPR spectrum considerably, in a manner similar to that brought about by addition of L-tryptophan, both in the line shape and the number of superhyperfine lines that could be observed (Fig. 2A and B, Spectra 4; and Table I).

From the comparison of integrated EPR signal intensities, in the absence of tryptophan, only 66% of the heme in tryptophan dioxygenase was bound to NO as compared to the protein in the presence of the substrate (Table II).

DISCUSSION

The description of the EPR spectra of tryptophan dioxygenase-NO alone, and in the presence of L-tryptophan, α-methyltryptophan, and 5-hydroxytryptophan, lead to several comments on the nature of the nitrosyl-heme complex and on the interaction between these sites, as seen locally by EPR, and the substrate binding sites on the regulator sites. First, the EPR spectra obtained in this study strongly suggest a nitrogen atom as the proximal ligand of the hemes in tryptophan dioxygenase. In fact, in the presence of substrate the superhyperfine pattern closely resembles those obtained with the nitrosyl derivatives of cytochrome c (9), α subunits of hemoglobin (11), and peroxidases (7, 10). As was demonstrated (7-9) this pattern arises from the interaction of the unpaired electron spin with the nuclear spin of 14N of NO (20 to 24 G coupling) and with the nuclear spin of the proximal nitrogen ligand of the heme (6.5 to 7.5 G coupling). In all the hemoproteins studied so far this proximal ligand is part of a protein amino acid residue: a histidine residue in the case of cytochrome c and α chains of hemoglobin and an as yet unknown residue in the case of peroxidases. In the presence of L-tryptophan or of 5-hydroxytryptophan, we cannot exclude completely that the superhyperfine structure of nine lines arises from the interaction with the nitrogen atom of tryptophan, now bound as a proximal ligand to the heme. This seems, however, highly unlikely because L-tryptophan bound at the catalytic site must at some point interact with oxygen bound on the distal side of the heme.

Second, we have described the symmetry of tryptophan dioxygenase-NO paramagnetic center as being rhombic, due to the three different principal g values found (Table I). In the presence of L-tryptophan the EPR spectrum observed was similar to those of cytochrome c (8), α subunits of hemoglobin (10), and catalase (7); $g_2$ was found, however, to have an unusual high value, 2.088. On the other hand, the in-plane feature in the $y$ direction coalesces with that in the axial ($z$) direction, as $g_2$ is equal to 2.004 and $g_3$ is barely resolved, close to 1.983; this is in contrast to the symmetry observed for horseradish (7) and turnip (10) peroxidases where $g_2$ is distinct from $g_3$ and is centered at $g = 1.96$. The similarity of the
Fig. 2. A, EPR spectra of the nitric oxide complex of ferrous tryptophan-2,3-dioxygenase in frozen solution at 77 K. 1, tryptophan dioxygenase-NO alone; 2, tryptophan dioxygenase-NO (35 μM) in the presence of L-tryptophan (2.5 mM); 3, tryptophan dioxygenase-NO (35 μM) in the presence of α-methyltryptophan (2.5 mM); 4, tryptophan dioxygenase-NO (35 μM) in the presence of α-methyltryptophan (2.5 mM) and of 5-hydroxytryptophan (1 mM). B, same conditions as in A, except for a 4-fold expanded field scale around g = 2.

### Table I

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<th>Compound</th>
<th>g&lt;sub&gt;1&lt;/sub&gt;</th>
<th>g&lt;sub&gt;2&lt;/sub&gt;</th>
<th>g&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Type</th>
<th>Number of lines</th>
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<th>A&lt;sub&gt;3&lt;/sub&gt;</th>
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<td>Tryptophan dioxygenase-NO</td>
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<tr>
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<td>1.988</td>
<td>B</td>
<td>3</td>
<td>16.5</td>
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<td>2.095</td>
<td>2.009</td>
<td>1.988</td>
<td>1.982</td>
<td>C</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>Tryptophan dioxygenase-NO + α-methyltryptophan + 5-hydroxytryptophan</td>
<td>2.089</td>
<td>2.040</td>
<td>2.004</td>
<td>1.983</td>
<td>B</td>
<td>3</td>
<td>16.5</td>
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*For a discussion of lineshapes of heme-NO complexes see Kon and Kataoka (9).*

spectra obtained for NO-cytochrome c, NO-α chains, and tryptophan dioxygenase-NO on the one hand, and the differences observed by comparison with nitrosyl-peroxidases on the other hand, allow us to suggest that the nitrogen atom proximal ligand of the hemes in tryptophan dioxygenase-NO arises from the imidazole of a histidine residue. Other evidence for such a proximal ligand was also derived from EPR studies of the ferric enzyme.

Third, the change of the symmetry of the paramagnetic center (9) upon addition of substrate, L-tryptophan, or its inhibitor, 5-hydroxytryptophan, is correlated with a modification of the superhyperfine structure from a pattern comprising essentially three lines (Fig. 2B, Spectra 1 and 3) to a pattern of nine regular lines (Spectra 2 and 4). These modifications reveal drastic alterations of the bonding of the iron atom with its two axial ligands, NO and the proximal nitrogen. Studies of model compounds (9, 13) allow the following interpretation.

Upon binding of L-tryptophan, the bond between the heme and the proximal nitrogen is shortened while that with NO is weakened. The rearrangement of the unpaired electron delocaliza-
TABLE II
Relative integrated signal intensity for nitrosyl tryptophan-2,3-dioxygenase prepared in presence of substrate and substrate analogs

In all experiments, the concentration of enzyme was 35 μM. The concentration of added compounds is indicated in each instance.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Relative signal intensity</th>
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<tr>
<td>None</td>
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<tr>
<td>α-Methyltryptophan (2.5 mM)</td>
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<tr>
<td>α-Methyltryptophan (2.5 mM) + 5-hydroxytryptophan (1 mM)</td>
<td>0.80</td>
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<td>L-Tryptophan (2.5 mM)</td>
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The inductive effect can be triggered by some ionization or by local movement due to the substrate or inhibitor binding.

Finally, the addition of substrate and effectors not only modifies the shape of the EPR absorption but also modifies its intensity indicative of extent of binding to NO (Table II). A quantitative evaluation of this phenomenon, which required double integrations of EPR signals of different shapes, showed that the EPR signals intensities increase in the following order: tryptophan dioxygenase, tryptophan dioxygenase + α-methyltryptophan, tryptophan dioxygenase + 5-hydroxytryptophan, tryptophan dioxygenase + tryptophan. The addition of tryptophan to tryptophan dioxygenase increases the EPR signal intensity by 50%, confirming the observation made on the optical absorption spectra that the addition of substrate increases the affinity of the enzyme for NO. This increase of affinity accompanied by the alteration of the superhyperfine pattern of the EPR from three to nine lines is reminiscent of the observation made on hemoglobin hybrids of the form CO-NO-deoxy (11) where oxygenation of the β chains produced the same type of spectral alterations. Although little is known yet on the intrinsic affinity of heme sites for ligands such as O₂, CO, or NO, it has been suggested (14) that in hemoglobin derivatives a correlation can exist between the affinity of ligand (CO, NO) and the number of superhyperfine lines seen in the EPR spectrum of the corresponding NO derivatives of these molecules. Further work is however needed to decide whether the number of superhyperfine lines can be generally taken as a probe of the affinity state of a hemoprotein for ligands to the heme site. Although EPR spectra cannot bring direct evidence on the structure of the substrate binding sites, our findings are compatible with the often proposed suggestion that α-methyltryptophan binds to a site different than the catalytic site where L-tryptophan and 5-hydroxytryptophan bind. Finally, our spectra do not distinguish structurally between the 2 hemes carried by the tetrameric enzyme.

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