^{1}H NMR spectra were obtained on the IBM-NR 80 spectrometer at 80 MHz with TMS as the internal standard. GC analyses were performed on a Perkin-Elmer 8410 instrument equipped with a column of 10% Carbowax 20M on Chromosorb WAW. ESR spectra were obtained on an IBM Instruments ESP-300 spectrometer at 9.602 GHz.

Synthesis of Substrates. *endo*-3-(*N*,*N*-Diethylamino)-6-methyl-*N*-phenyl-2,3-oxaphosphabicyclo[2.2.2]oct-5-ene-8,9-dicarboximide 3-oxide (**1**),[16] *endo*-3-ethoxy-6-methyl-*N*-phenyl-2,3-oxaphosphabicyclo[2.2.2]-oct-5-ene-8,9-dicarboximide 3-oxide (**6**),[1] and *endo*-3-ethoxy-6-methyl-*N*-phenyl-2,3-oxaphosphabicyclo[2.2.2]oct-5-ene-8,9-dicarboximide 3-sulfide (4)[2] were prepared as previously reported. The compounds were purified by column chromatography followed by several recrystallizations.

Synthesis of *N*-Phenyl-4-methyl-1,2-dihydrophthalimide (3). The bicyclic phosphonamide **1** (0.345 g, 0.92 mmol) and ethanol (0.35 mL, about 5 mmol) in 5 mL of dry $CHCl_3$ were heated in a closed ampule for 12 h at 100 °C. The product mixture was separated by column chromatography with CH_2Cl_2/hexane (2:1) as eluant. The eluate containing **3** was evaporated to dryness in vacuo to give a white solid whose ^{1}H NMR spectrum and mp (120.5-123 °C) were the same as were reported (lit.[26] mp 120-123 °C).

Solvents for Kinetics Experiments. DMSO was dried over molecular sieves and then fractionally distilled in vacuo. Chloroform, acetonitrile, and benzonitrile were fractionally distilled over P_2O_5. Toluene was dried by boiling over sodium and then fractionally distilled. Tetrahydrofuran was dried with $MgSO_4$ and then molecular sieves; it was further purified on an Al_2O_3 column and distilled over potassium.

Kinetics Measurements. The rate of disappearance of substrate was determined from the diminution of its ^{31}P NMR signal. Into a 10-mm NMR tube was placed a coaxial sealed 5-mm NMR tube containing D_2O or a $CDCl_3$ solution of a phosphorus standard ($(EtO)_2P(O)OH$ or $(EtO)_2P(O)Me$). A solution (2 mL) of the substrate (usually 0.2 mmol) was added and the external tube was sealed under argon. The assembly was placed in the vapor space of a 3-L flask containing a refluxing solvent (methanol, 65 °C; benzene, 80 °C; trichloroethylene, 87 °C; 2-propanol, 97 °C; dioxane, 101 °C; toluene, 110 °C). The temperature was constant within ±0.1 °C. At various time intervals (usually eight) the tube was removed, cooled, and the ^{31}P NMR spectrum recorded. The concentration of the remaining substrate was determined from peak area-concentration plots that were prepared for the $(EtO)_2P(O)OH$ or $(EtO)_2P(O)Me$ standards. In some experiments, an alcohol trapping agent was added to the solution of the substrate.

Calculations of the rate constants and the Arrhenius parameters were carried out by the least-squares method, and all data are given with standard deviations. The enthalpy and entropy of activation were calculated for 100 °C according to Eyring theory.

Photochemical Fragmentation. These experiments were carried out in a Rayonet photochemical reactor fitted with 16 low-pressure mercury lamps (253.7 nm). The light intensity was determined with a potassium ferrioxalate actinometer[27] and was found to be 13.2×10^{-7} einstein min^{-1} mL^{-1}. A quartz NMR tube containing 2 mL of a solution of **1** or **4** (occasionally with an alcohol as a trapping agent) was placed in a quartz thermostat with distilled water as the cooling medium (T = 28-30 °C). The thermostat was placed in the center of the UV reactor. The solution was flushed with dry argon during irradiation. To determine the change of concentration of the substrate, ^{31}P NMR peak areas were compared to a deuteriochloroform solution of $(EtO)_2P(O)OH$ as a standard in an internal sealed tube. The same procedure was used in competition experiments with metathiophosphate **5**. Data are recorded in Table VII.

Alcohol Competition Experiments. The ratios of the rate constants for reactions of mixtures of ethanol and another alcohol with metathiophosphate **5** and metaphosphate **7** were calculated according to eq 4.

$$k_{EtOH}/k_{ROH} = \frac{[(EtO)_2P(X)OH]}{[(EtO)(RO)P(X)OH]}\frac{[ROH]}{[EtOH]} \quad (4)$$

The sample of the solution of precursor **4** or **6** and the alcohol mixture was heated for about 2 half-lives (70% completion). The ratios of concentrations of phosphates were found from the ratios of the ^{31}P NMR integrations of these products. GC was used to determine the ratios of concentrations of the alcohols. Competition experiments were also performed with **4** when photochemically generated. Data are presented in Table VI.

Acknowledgment. This work was supported by a grant from the Army Research Office. S.J. thanks the Technical University of Lodz, Poland, for a leave of absence.

(26) Shusherina, N. P.; Nesterova, T. L.; Polyakova, O. V. *Zh. Org. Khim.* **1980**, *16*, 1285 (*J. Org. Chem. USSR*, *16*, 1111).

(27) Hatchard, C. G.; Parker, C. A. *Proc. R. Soc. London A* **1956**, *235*, 518.

Conformational and Dynamic Changes of D- and L-Tryptophan Due to Stereoselective Interaction with Human Serum Albumin, As Revealed by Proton-Selective Relaxation Rate Measurements†

Gloria Uccello-Barretta, Carlo Bertucci, Enrico Domenici,‡ and Piero Salvadori*

Contribution from the Dipartimento di Chimica e Chimica Industriale, Centro di Studio del CNR per le Macromolecole Stereordinate ed Otticamente Attive, via Risorgimento 35, 56126 Pisa, Italy. *Received July 26, 1990*

Abstract: The proton selective relaxation rates of D- and L-tryptophan are affected to a different extent by the interaction with human serum albumin. These differences are correlated to a different degree of immobilization of the two enantiomers at the protein binding site. Conversely, no differences are detected in their intermolecular dipolar interaction with the protein protons.

Introduction

The growing interest in the relationship between stereochemistry and biological activity requires the development of methods for elucidating the interaction mechanism of chiral molecules with biological substrates. A number of methods have been proposed for the study of interactions between small ligands and macromolecules, such as substrate-enzyme or drug-protein interactions.[1,2] Nuclear magnetic resonance (NMR) methods based on

† Dedicated to the memory of Professor Piero Pino.

‡ Present address: Microbiology Department, Glaxo Research, via Fleming 4, 37100 Verona, Italy.

(1) Cantor, C.; Schimmel, P. R. *Biophysical Chemistry*, Parts II and III; Freeman, W. H., Ed.; San Francisco, 1980.

(2) Jardetzky, O.; Roberts, G. C. K. *NMR in Molecular Biology*; Academic Press: New York, 1981.

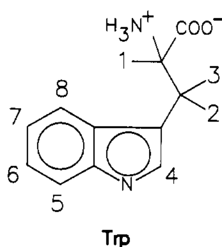
0002-7863/91/1513-7017$02.50/0 © 1991 American Chemical Society

Table I. Observed Proton-Selective Relaxation Rates (R^s_{obs}, s^{-1}) for Trp in the Free State and in D-Trp/HSA and L-Trp/HSA Mixtures ([Trp] = 0.03 M; Trp/HSA = 140; D_2O; 22 °C; pH 7.4)

	free	D-Trp/HSA	L-Trp/HSA
H^1	0.39 ± 0.02	1.50 ± 0.03	1.27 ± 0.04
H^2	1.10 ± 0.02	3.73 ± 0.03	2.99 ± 0.03
H^3	1.12 ± 0.02	3.69 ± 0.03	3.19 ± 0.02
H^4	0.15 ± 0.07		
H^5	0.14 ± 0.01	0.92 ± 0.02	0.75 ± 0.02
H^6	0.39 ± 0.03	2.00 ± 0.05	1.44 ± 0.05
H^7	0.39 ± 0.03	2.02 ± 0.03	1.55 ± 0.04
H^8	0.37 ± 0.03	1.80 ± 0.02	1.41 ± 0.02

the determination of the relaxation rate of selectively excited protons[3-5] have been extensively employed in detecting the conformational and dynamic features of small molecules interacting with proteins.[6,7]

Preliminary results[8] have shown the potential of selective relaxation rates in detecting the stereoselective interactions occurring in solution between the two antipodes of tryptophan (Trp) and the binding site of human serum albumin (HSA).[9-11]



Trp

We report here a detailed analysis of the proton selective relaxation parameters of D- and L-Trp in the free state and in the presence of HSA. The aim of the work is to demonstrate the applicability of this method in detecting differences in the dynamics and stereochemistry of the two enantiomers in the presence of HSA.

Results and Discussion

Methods based on the determination of the selective relaxation rates (R^s) take advantage of the favorable dependence of R^s on the correlation time in the region of slow molecular motions, in which the small molecule is forced by the interaction with the macromolecule.[7] In the fast-motion region ($\omega\tau_c \ll 1$; ω = Larmor frequency, τ_c = reorientational correlation time), both the selective and nonselective (R^{ns}) relaxation rates increase progressively with increasing τ_c. When the molecular motion of the ligand is slowed to the $\omega\tau_c \gg 1$ region as a consequence of the interaction with the macromolecule, R^s shows a sharp increase, whereas R^{ns} reaches a maximum for $\omega\tau_c \approx 1$ and then decreases with further increasing $\omega\tau_c$. Therefore, under these last conditions and in the fast-exchange limit, only variations of R^s are detectable, particularly in the presence of a large excess of free ligand with respect to the bound one. We found that the relaxation rates of aromatic and alkyl protons of Trp show a remarkable increase in the mixtures D-Trp/HSA and L-Trp/HSA. The data suggest that the binding with HSA involves hydrophobic interactions via the aromatic moiety of Trp and electrostatic interactions via its amino and carboxyl groups. Interestingly enough, in spite of the high molar ratio of Trp/HSA used (140) (Table I), the presence of HSA affects the monoselective relaxation rate of each proton of the two enantiomers to a different extent. Greater R^s values (R^s_{obs}) are measured for each proton of D-Trp relatively to L-Trp (Table I). As an example, the selective relaxation rate of the aromatic proton named H_8 is 0.37 s^{-1} in the free Trp, but in the presence of HSA (Trp/HSA = 140), it increases remarkably to 1.80 s^{-1} for the D form and to 1.41 s^{-1} for the L form.

Table II. Proton Monoselective Relaxation Rates of D-Trp and L-Trp in the Bound State (R^s_{bound}, s^{-1}) ([Trp] = 0.03 M; Trp/HSA = 140; pH 7.4; D_2O; 22 °C)

	D-Trp	L-Trp		D-Trp	L-Trp
H^1	155.4	123.2	H^5	109.2	85.4
H^2	368.2	264.6	H^6	225.4	147.0
H^3	359.8	289.9	H^7	228.2	162.4
H^4			H^8	200.2	145.6

Table III. Cross-Relaxation Terms (σ_{ij}, s^{-1}) for Trp in the Free and Bound States ([Trp] = 0.03 M; Trp/HSA = 140; pH 7.4; D_2O; 22 °C)

	free	D-Trp/HSA	L-Trp/HSA
σ_{23}	0.470	−140.0	−107.8
σ_{56}	0.070	−36.4	−26.6
σ_{78}	0.069	−36.2	−26.5

In the fast-exchange limit, the measured relaxation rates (R^s_{obs}) are the weighted means of the values in the bound (R^s_{bound}) and free (R^s_{free}) states

$$R^s_{obs} = x_{bound}R^s_{bound} + x_{free}R^s_{free}$$

where x_{bound} and x_{free} are the molar fractions of the bound and free Trp, respectively. At the high molar ratios of Trp/HSA used, the molar fraction of Trp bound is negligible compared with that of free Trp and the relaxation rates for D- and L-Trp in the bound state (R^s_{bound}) can be easily evaluated, assuming x_{free} = 1 and x_{bound} = 1/140 (and assuming a 1/1 interaction) (Table II).

As an example, R^s_{bound} of the aromatic proton H_8 is 200.2 s^{-1} for D-Trp and 145.6 s^{-1} for L-Trp. Therefore, the R^s value of this proton shows about 500-fold increase in the bound state with respect to the free state, whereas a 54.6-s^{-1} difference is found in the relaxation rates of the two bound enantiomers. This last difference, though small if referred to the difference observed between the R^s values of Trp in the free and bound states, is completely reproducible.

Since the NMR parameters observed do not depend on the molar ratio of Trp/HSA (in the range between 30 to 140), the differences between R^s_{bound} and R^s_{free} are not attributable to increased viscosity nor to aspecific interactions. It is important to point out that under the conditions in which the experiments were carried out the specific binding site was reasonably well saturated. Therefore, the differences between the proton relaxation rates of the two enantiomers in the bound state cannot be attributed to the difference between the dissociation constants of the two complexes D-Trp/HSA and L-Trp/HSA. Indeed, at the high molar ratio of Trp/HSA used, the bound fractions of the two enantiomers are almost equal, as calculated from the corresponding dissociation constants.[10]

In order to gain deeper insight into the origin of the observed differences between the R^s values of the two enantiomers in the bound state, we have used a combination of biselective (simultaneous excitation of one proton pair ij) (R^i_{ij}) and monoselective (excitation of only one proton i) (R^i) relaxation rate measurements to calculate the cross-relaxation term σ^i_{ij} for the proton pair ij. This parameter is a relatively simple function of the interproton distance r_{ij} and of the correlation time τ_{cij} of the vector ij.

(3) Freeman, R.; Hill, H. D. W.; Tomlinson, B. L.; Hall, L. D. *J. Chem. Phys.* **1974**, *61*, 4466–4473.
(4) Noggle, J. H.; Schirmer, R. E. *The Nuclear Overhauser Effect*; Academic Press: New York, 1971.
(5) Neuhaus, D.; Williamson, M. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; VCH Publishers: New York, 1989.
(6) Valensin, G.; Kushnir, T.; Navon, G. *J. Magn. Reson.* **1982**, *46*, 23–29.
(7) Valensin, G.; Sabatini, G.; Tiezzi, E. In *Advanced Magnetic Resonance Techniques in Systems of High Molecular Complexity*; Niccolai, N., Valensin, G., Eds.; Birkhauser: Boston, 1986; pp 69–76.
(8) (a) Uccello-Barretta, G.; Bertucci, C.; Domenici, E.; Salvadori, P. In Proceedings of the Palio Workshop on Molecular Structure Dynamics and Recognition; Siena, Italy, 1988; pp 70–71. (b) Uccello-Barretta, G.; Domenici, E.; Bertucci, C.; Salvadori, P. *J. Chem. Soc., Chem. Commun.* **1989**, 1178–1179.
(9) Kragh-Hansen, U. *Pharmacol. Rev.* **1981**, *33*, 17–53.
(10) McMenamy, R. H.; Oncley, J. L. *J. Biol. Chem.* **1958**, *233*, 1436–1447.
(11) Fitos, I.; Simonyi, M. *Acta Biochim. Biophys. Hung.* **1986**, *21* (3), 237–245.

$$\sigma^i_{ij} = R^i_{ij} - R^i$$

$$\sigma^i_{ij} = 0.1\gamma^4 h^2 r_{ij}^{-6}[6\tau_c/(1 + 4\omega^2\tau_c^2) - \tau_c]$$

In this way we have calculated the cross-relaxation terms for the proton pairs 7–8, 5–6, and 2–3 (Table III), which are 0.069, 0.070, and 0.470 s^{-1}, respectively, for the free Trp; these values change to −36.2, −36.4, and −140 s^{-1} in the D-Trp/HSA mixtures, while for the mixtures L-Trp/HSA they are −26.5, −26.6, and −107.8 s^{-1}, respectively. The changes in the sign of the cross-relaxation terms in the bound states with respect to the free Trp indicate that the binding interaction with HSA slows the motion of the two antipodes to the $\omega\tau_c \gg 1$ region.[5] Since the interproton distances 5–6, 8–7, and 2–3 are the same in the two enantiomers, the differences found in the cross-relaxation rates of the two bound enantiomers are related to differences in their dynamic properties. Therefore, we have calculated the reorientational correlation times of the vectors 7–8, 5–6, and 2–3 for the amino acid in the free and bound states, from the known values of the interproton distances[12,13] r_{78}, r_{56}, and r_{23}. The motion of free Trp is completely isotropic, since the reorientational times calculated for the three vectors are nearly identical: 4.95×10^{-11} for the aromatic moiety and 4.85×10^{-11} for the alkyl chain. A remarkable change is found in the D-Trp/HSA mixture, where the reorientational time of the 7–8 and 5–6 vectors increases to 1.29×10^{-7} s, whereas for the vector 2–3 a value of 7.22×10^{-8} s is found. Therefore, the aromatic moiety is immobilized by the interaction with HSA to a greater extent than the alkyl chain. Different correlation times are calculated for L-Trp in the bound state: these are 9.41×10^{-8} s for the vectors 7–8 and 5–6 and 5.55×10^{-8} s for the vector 2–3. The above data indicate that the molecular motion of D-Trp is slowed more than that of L-Trp. However, for both enantiomers the reorientational time of the aromatic moiety is longer than that of the alkyl chain and the ratio of τ_{c87} or τ_{c56} to τ_{c23} is the same for the two enantiomers. The monoselective relaxation rate of one proton i (R^i) is given by the pairwise summation, over all the proton pairs ij, of the direct intramolecular dipole–dipole terms (ρ_{ij}) and by a term ρ^*, which is contributed by other sources of relaxations (mainly intermolecular dipole–dipole interactions)[5]

$$R^i = \sum \rho_{ij} + \rho^*$$

where

$$\rho_{ij} = 0.1\gamma^4 h^2 r_{ij}^{-6}[3\tau_c/(1 + 4\omega^2\tau_c^2) + 6\tau_c/(1 + 4\omega^2\tau_c^2) + \tau_c]$$

Thus, the reorientational time τ_{cij} (obtained from the experimental cross-relaxation terms σ_{ij}) can be used to calculate the ρ_{ij} terms for each proton pair ij having a known interproton distance r_{ij}. The pairwise summation of all the two spin terms ρ_{ij} can be used to calculate the contribution to the relaxation rate of the proton i, due to intramolecular interactions with j protons.

As far as the proton H_5 is concerned, its monoselective relaxation rate contains contributions from intramolecular dipole–dipole interaction with the proton H_6 and, for the two bound isomers, from intermolecular interaction with the protein:

$$R^5 = \rho_{56} + \rho^*$$

The direct term ρ_{56}, calculated by using the τ_c values of the 5–6 vector, is 36.4 s^{-1} for D-Trp bound, 26.6 s^{-1} for L-Trp bound, and 0.135 s^{-1} for free Trp. This last value is nearly equal to R^5, as expected when the relaxation arises completely from the intramolecular dipole–dipole interactions with the proton H_6. For the two bound isomers, only a minor part of the relaxation (about 30%) is due to intramolecular interaction with H_6. The difference between R^5 and ρ_{56} can be reasonably regarded as an indication of the extent of protein interaction.

By extending the calculation to the proton H_6, very similar results are obtained. The intramolecular part of the relaxation ($\rho_{65} + \rho_{67}$), arising from the interaction with H_5 and H_7, is 0.34 s^{-1} for the free substrate, 70 s^{-1} for D-Trp, and 52 s^{-1} for L-Trp bound. As is found for H_5, the relaxation of H_6 in the absence of the protein is due only to intramolecular mechanism, whereas binding to HSA reduces this contribution to about 30%.

In the case of the proton H_8, on considering its intramolecular relaxation as mainly due to the dipolar interaction with the proton H_7, the intramolecular part of R^8 is given only by the term ρ_{87}, which is calculated as 36.4 s^{-1} for D-Trp bound and 26.6 s^{-1} for L-Trp bound. Therefore, for this proton, the intramolecular contribution is significantly lower (18%) than that noted earlier for the proton H^5. On the other hand, a similar result was obtained for the free Trp, where no intermolecular contribution to the relaxation is expected. Here, the ρ_{87} term is 0.135 s^{-1}, which is only 50% of the measured selective relaxation rate R^8 (0.37 s^{-1}). A reasonable explanation of the above results could be the following: as a consequence of folding of the alkyl chain, the intramolecular relaxation of H_8 is due not only to the interaction with the proton H_7 but also to the interaction with the alkyl chain protons. If this is so, a complete analysis of R^8 would require the evaluation of additional ρ_{8i} terms (i = 1–3).

Conclusions

Proton-selective relaxation parameters of D- and L-Trp in the presence of HSA reflect a different degree of immobilization of the enantiomers at the protein binding site. No differences are detected in the relative contribution to the relaxation by intermolecular dipolar interaction with the protein protons. According to previous results obtained by T_2 measurements,[14] L-Trp, which has a higher affinity to HSA relatively to D-Trp, is the antipode perturbed to a lesser extent. It could be due to the fact that the L enantiomer fits the stereochemical constraints at the binding site better than the D enantiomer does.

The measurement of selective relaxation rates has been widely used to study the interaction of small ligands with biopolymers.[6,7] It is noteworthy that this method is here successfully applied, for the first time, to monitor differences in the interaction of two antipodes in the bound state. Since differences in the selective relaxation rates of the two bound enantiomers are related to their conformation and dynamics, the origin of the stereoselectivity occurring at the protein binding sites can be investigated.

Experimental Section

Selective relaxation rates were measured in the initial rate approximation on a Varian VXR-300 MHz instrument by applying a selective 180° pulse with the proton decoupler at the selected frequency for 20 ms. After time t, a nonselective 90° pulse was applied to detect the longitudinal magnetization. Measurements were carried out for all protons of L- and D-Trp in the free state and in the presence of HSA ([Trp] = 0.03 M; Trp/HSA = 140, 70, 35; pH 7.4 in phosphate buffer, D_2O solutions, 22 °C). Each measurement was repeated at least four times. Oligomerized HSA was removed by gel filtration on a Sephacril S-300 column, 50 × 2.5 cm; monomeric HSA was lyophilized and then dissolved in the deuteriated buffer.

Registry No. D-TRP, 153-94-6; L-TRP, 73-22-3.

(12) Yamane, T.; Andou, T.; Ashida, T. *Acta Crystallogr.* **1977**, *B33*, 1650–1653.
(13) Gartland, G. L.; Freeman, G. R.; Bugg, C. E. *Acta Crystallogr.* **1974**, *B30*, 1841.

(14) Monti, J. P.; Sarrazin, M.; Briand, C.; Crevat, A. *J. Chim. Phys.* **1977**, *74*, 942–946.