Complex of Calcium Receptor Blocker Nifedipine with Glycyrrhizic Acid

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Physicochemical methods were used to explore the regularities of complexing between the calcium channel blocker nifedipine (NF) and pharmaceutically acceptable complex-forming glycyrrhizic acid (GA) in view of the discovered influence of GA on the therapeutic activity of NF. ^{1}H NMR (including relaxation measurements) and UV−vis spectra have produced illustrative evidence that NF forms stable complexes with GA within a wide concentration range, from 0.05 to 5 mM. At low GA concentrations, below 0.5 mM, NF forms an inclusion complex where each NF molecule is bound by two molecules of GA. Computer simulations of the NMR experimental data have shown that, in aqueous solution, the stability constant of this complex, *K*, is about 10^5 M^{-1}. At higher concentrations, GA forms large micelle-like aggregates which increase the water solubility of NF. Quenching of chemically induced dynamic nuclear polarization effects in the photoinduced interaction of the NF−GA complex with tyrosine suggests that complex formation with GA completely blocks the single electron-transfer step between NF and the amino acid. This, arguably, could explain the increased therapeutic activity of GA complexes, since GA might protect the drug molecule from the reaction with amino acid residues of the receptor binding site.

Introduction

During the past decade physicochemical approaches are increasingly employed to explore the detailed mechanisms of biologically relevant processes. One of the most challenging tasks of modern pharmaceutical science involves the attempts to increase a drug's efficiency, at the same time, lowering its toxicity. A promising way to create novel and more powerful forms is the preparation of molecular complexes of the existing drugs with known complexing agents. Such agents are able to facilitate drug delivery, to protect parent substances from metabolic decay, and to contribute to their prolonged action. β-Glycyrrhizic acid (GA), a triterpene glycoside from *Glycyrrhiza uralensis fisher* (Fabaceae), is one of the most promising complexing agents that was recently demonstrated to reduce toxicity and to increase therapeutic activity of molecular complexes formed by several important antibiotics and cardiovascular preparations.[1−4] For example, the complexes of GA with nifedipine and lappaconitine, an antiarrhythmic diterpene alkaloid from *Aconitum septentrionale koelle* (Ranunculaceae), show significant increase in their therapeutic activity on the models of adrenaline-induced hypertension[3] and $CaCl_2$ induced arrhythmia.[4] From our viewpoint, to understand the reasons underlying the above-mentioned interplay between toxicity and therapeutic activity, one should explore the molecular-level mechanisms of the impact of complexing on drug activity.

Most recently, the issue of the molecular-level reaction mechanisms which might occur between a drug and its binding site in the membrane-integrated receptor protein is coming to the forefront, gradually replacing the traditional macroscopic approach of donor−acceptor formation and decay of ligand−receptor complex. Apart from its evident fundamental significance, the understanding of the mechanisms of molecular-level drug−receptor interactions might be expected to become the determinant in the development of new drug preparations. The knowledge of fine details of chemical reactions defining binding and dissociation of the drug−receptor complex will allow one to understand the driving force of the therapeutic activity, to propose methods to control the influence of the drug, and to minimize possible side effects.

In our earlier article, we have described the potential model of drug−receptor interaction where chemical transformations of the drug were a key step in the dissociation of the drug−receptor complex.[5,6] The model was evaluated using the calcium channel blocker nifedipine[5] and the above-mentioned antiarrhythmic alkaloid lappaconitine[6] (Lap). Nifedipine (NF, 1,4-dihydro-2,6-dimethyl-4-(2′-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester), one of the commonly used dihydropyridine derivatives, forms a complex with the binding site of the L-type calcium receptors comprised of six spatially separated amino acid residues when its conformation corresponds to the closed channel.[7] Since detailed computational analysis[8] demonstrated noticeable charge transfer between tyrosine residual and NF in the receptor's binding site, it was reasonable to consider the single electron transfer as the potentially most probable mechanism of the NF interactions with the environment of the Ca^{2+} receptor's binding site.[5] We have suggested that the complexing might also affect the efficiency of the above-mentioned step of chemical transformations of the drug bound to the receptor. Decrease in the rate of the drug−receptor interaction, i.e., the rate of the transformation of the drug into reaction product as a result of the interaction with amino acid residues of the receptor's binding site, might prolong the effective retention time of the drug linked to the membrane receptor. This assumption has been confirmed in the experiments involving the complexes of GA with Lap.[9] It has been shown that, in this case, complexing leads both to a decrease of minimum

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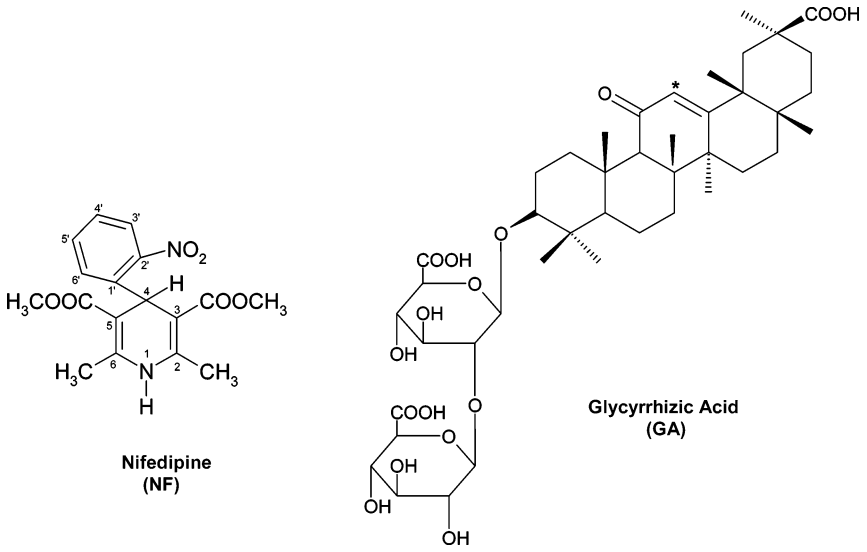
SCHEME 1: Structural Formulas of Nifedipine (NF) and Glycyrrhizic Acid (GA)



therapeutic dose of the drug and to a deceleration of the reaction rate between Lap and amino acids forming the receptor's binding site.[9] Another hypothesis can be drawn from the recently reported strong influence of GA addition on the HSA (human serum albumin) binding of NF.[10] Since GA protects NF from binding to HSA, this can result in significant increase of free NF concentration.

The present article describes the attempts to develop a physicochemical approach which (i) combines the advanced physicochemical methods to demonstrate the existence of the complex of NF with GA, (ii) considers the influence of complexing with GA on the efficiency of single electron transfer between NF and tyrosine as follows from chemically induced dynamic nuclear polarization (CIDNP) experiments, and (iii) draws a correlation between therapeutic activity and physicochemical characteristics of the complex.

Experimental Section

Chemicals and Preparation of the Complex. Nifedipine (Sigma) and *N*-acetyl tyrosine (Tyr, Sigma) were used as supplied. Glycyrrhizic acid was kindly donated by Professor N. F. Salakhutdinov (Novosibirsk Institute of Organic Chemistry, Novosibirsk). For NMR experiments, D_2O and CD_3OD (Aldrich), or their mixtures (v/v), were used as solvents. Since both NF and GA have extremely low solubility in pure water, all experiments were carried out in water−methanol mixtures. NF−GA complex was prepared by addition of methanol solutions of NF and GA to a water aliquot followed by stirring for several hours. After standing in the dark overnight, the clear solution was decanted and analyzed. The total methanol fraction in solution used to study the solubility diagram was equal to 5%. The concentration of GA was varied from 0 to 4 mM.

Physicochemical Study of the Complex. The solubility of NF was monitored by optical density of the solution at the absorption maximum of NF at 315 nm ($\epsilon_{315} = 7500$). To measure the stoichiometry of the complex and its stability constant, we have employed a standard NMR procedure[11,12] which considers the variation of chemical shifts of GA protons (denoted by (★) in Scheme 1) depending on the concentration of NF. NMR spectra were recorded using a Bruker DPX-200 NMR spectrometer (200 MHz ^{1}H operating frequency). Note that, in this case, the variation range of chemical shifts induced by complex formation (several hertz) is much lower as compared to the one observed for the widely used cyclodextrin complexes.[11] This hampers the use of the standard NMR technique for the investigation of the complexes formed by GA. T_2 relaxation was measured by means of the Carr−Purcell−Meiboom−Gill pulse sequence:[13] $p(90°) - (\tau - p(180°) - \tau)_n$ − acquisition, where $\tau = 6$ ms and n was varied from 0 to 4028. The time dependence of the NMR signal intensity is described by exponential function 1.

$$I(t) = I_o \exp\left(-\frac{\tau}{T_2}\right) \quad (1)$$

T_2 values were calculated by fitting experimental data using formula 1.

Chemically Induced Dynamic Nuclear Polarization Experiments. In CIDNP experiments, the samples in standard 5 mm Pyrex NMR tubes were irradiated directly in the probe of the NMR spectrometer at room temperature. An EMG 101 MSC Lambda Physik excimer laser was used as the light source ($\lambda = 308$ nm, pulse duration 15 ns, average pulse energy 100 mJ). Time-resolved (TR) CIDNP spectra were detected using a DPX 200 Bruker NMR spectrometer (200 MHz ^{1}H operating frequency) using a standard presaturation technique to suppress the equilibrium signals. The pulse sequence included (i) saturating radiofrequency pulse, (ii) laser pulse, (iii) delay time, (iv) detecting radiofrequency pulse, and (v) free induction decay. Because of the suppression of the background (equilibrium) NMR signals in the CIDNP spectrum, only the lines of the products demonstrating nuclear polarization could be observed.

Results and Discussion

The influence of complexation on a set of physicochemical properties of the compounds bound in a complex is widely used both as an evidence of complex formation and for its characterization and in the applications designed to alter and/or control selected chemical processes. Drug complexes are typically prepared with the purpose of increasing their overall stability and solubility in aqueous media. Since NF is insoluble in water and very slightly soluble in water−alcohol mixtures, we have attempted to prove the formation of the complex of NF with GA by two different methods: by examining its solubility dependence on the GA concentration and by measuring the spin−spin relaxation time T_2 of NF protons in the absence and

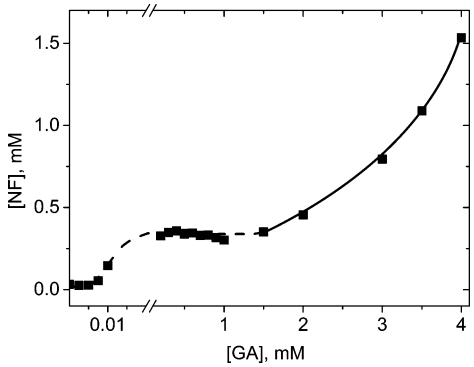


Figure 1. Solubility diagram of nifedipine (NF) as a function of the concentration of glycyrrhizic acid (GA) in aqueous solution containing 5% methanol.

in the presence of GA. The longitudinal, or spin−lattice, relaxation rate ($1/T_1$) and the transverse, or spin−spin, relaxation rate ($1/T_2$) are NMR parameters that may additionally be used to measure binding. T_1 or T_2 measurements are most likely to be useful when complexation-induced alterations of the chemical shifts are too small to be significant. This is exactly the case of GA complexes.

Solubility Diagram. To plot the solubility diagram the concentration of NF was monitored by optical density of the resulting solution of NF−GA complexes after stirring the mixture of NF and GA methanol solutions in the corresponding water aliquot for several hours followed by standing overnight and decanting (Figure 1). Measurements were performed within a wide range of GA concentrations (from 0 to 4 mM). Three characteristic regions are evident from the solubility diagram: (i) an increase of the NF concentration, (ii) a plateau, and (iii) the subsequent concentration growth. The observed solubility pattern allows us to suggest that the variation of GA concentrations results in the formation of differently organized complexes. It is noteworthy that a similar concentration dependence was also observed when studying the structure of GA solutions and the parameters of its flow dynamics at different concentrations.[14] In particular, it has been shown that the dynamic viscosity of the solution abruptly changes at certain concentrations of GA which might be stipulated by the variations in the structure of aggregates formed by GA molecules. Remarkably, the concentration points in the solubility diagram where step changes in the solubility of the NF in complex with GA are observed (Figure 1) corresponded to the earlier detected jump-in points in the dynamic viscosity of GA solutions[14] and the fluorescence intensity of carotenoids in GA complex.[15] Our recent NMR study of GA solutions has demonstrated the formation of GA micelles at concentrations higher 1 mM.[16] The present study shows that micelle formation results in further increase of NF solubility.

Of note, salient points of the solubility diagram could be regarded only as an indication of the formation of the NF−GA complex, since the respective concentration values in the curve very roughly reflect the ratio between the components of the complex. Instead, Job's plots (see below) provide much better data on the stoichiometry of the formed complexes.

***T_2* Relaxation Measurement.** Relaxation measurements were performed in pure and 30% methanol solutions at GA concentration equal to 0.7 mM, below the critical micelle concentration (cmc) point (~1 mM).[16] Figure 2 shows decay kinetics of the echo signal intensity of 2,6-CH_3 NF protons in pure methanol (1), in pure methanol in the presence of 0.7 mM GA (2), and in 30% methanol−water in the presence of 0.7 mM GA (3).

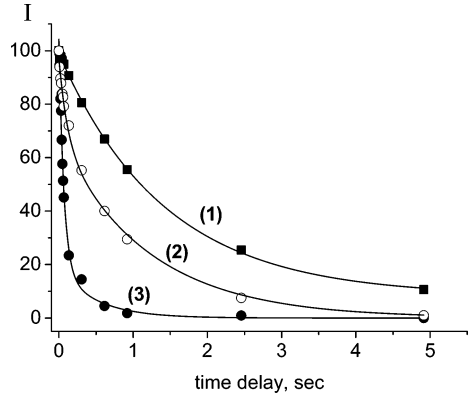


Figure 2. Changes in the echo signal intensity (I) of 2,6-CH_3 protons of NF vs time delay t ($t = 2\tau n$, see the Experimental Section) in pure methanol (1), in pure methanol in the presence of 0.7 mM GA (2), and in 30% methanol−water in the presence of 0.7 mM GA (3).

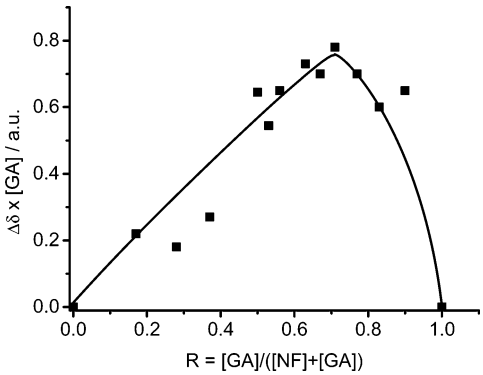


Figure 3. Job's plots corresponding to the changes in NMR chemical shifts of protons of glycyrrhizic acid (GA), δ 5.53 and 1.05 ppm, in the NF−GA complex in 30% CD_3OD/D_2O solution. [NF] + [GA] = 0.5 mM.

The experimental data for the pure methanol solution in the absence of GA are described by a one-exponential function (eq 1) with $T_2 = 1.42 \pm 0.035$ s. The decay kinetics of the NMR signal intensity versus time delay for the methyl protons of NF in the presence of GA were satisfactory fitted only by a two-exponential function (eq 2):

$$I(t) = I_1 \exp\left(-\frac{\tau}{T_{21}}\right) + I_2 \exp\left(-\frac{\tau}{T_{22}}\right) \qquad (2)$$

Here, T_{21} is the spin−spin relaxation time of free NF, T_{22} is the spin−spin relaxation time of NF in the complex, and I_1, I_2 (normalized in such a way that $I_1 + I_2 = 1$) are the fractions of free and complexed NF, respectively. This assumes a low exchange rate between free and complexed NF (as compared to $1/T_2$). Relaxation parameters (I_1, I_2, T_{21}, and T_{22}) calculated from eq 2 are summarized in Table 1.

These data show that complexation significantly reduces the relaxation time of bound molecules. With the known stoichiometry of the complex one can estimate the value of stability constant K_{1n} using the standard definition formula (eq 3):

$$K_{1n} = \frac{[\mathrm{NF} - \mathrm{GA}]}{[\mathrm{NF}][\mathrm{GA}]^n} \qquad (3)$$

Stoichiometry Measurement. Stoichiometry of the complex could be calculated from the dependence of the chemical shifts of the GA signals on the NF concentration (Job's plot).[13] Measurements were performed for a low GA concentration of 0.5 mM. The results are shown in Figure 3.

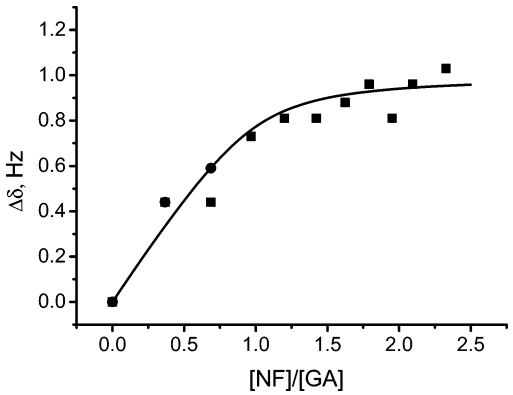


Figure 4. Analysis performed for the complex of the dimer of glycyrrhizic acid (GA) and nifedipine (NF) of 1:1 stoichiometry in 20% CD_3OD/D_2O solution, [GA] = 0.25 mM. Experimental concentration dependence (■) of the changes in the NMR chemical shift of CH_3 protons of GA, δ 1.05 ppm, on the addition of NF and its computed fit (—).

For the complex of NF with GA, the location of the maximum in the Job's plots (Figure 3) defines the stoichiometry of the complexes, 1:2 (NF/GA) at GA concentrations up to 0.5 mM. The above-mentioned structural studies of GA solutions at different concentrations[14] have suggested that, in solution, GA molecules form cyclic aggregates which have a structure dependent on the GA concentration. Therefore, one might assume that in the case of low GA concentrations, one molecule of NF forms a complex with a cyclic dimer of GA. A similar conclusion on the formation of cyclic dimers of GA has been drawn from the detailed study of GA complexes with carotenoids.[15,17] It has been shown that the increase of GA concentrations above 1 mM not only alters the structure of a complex but also results in the significant decrease of its stability constant.

In the present study, the stability constant of the NF−GA complex was measured by means of a routine NMR technique often used to analyze inclusion complexes, e.g., with cyclodextrins.[12,13] Additional information on the use of NMR to measure stability constants could be found in the comprehensive review.[18] In the present work, to measure the stability constant, we have considered the dependence of chemical shifts of the GA signal on NF concentration, at a GA concentration 0.25 mM. In general, the calculations of the stability constant for a complex are rather cumbersome, and a simple analytical solution (eq 4) could be drawn only for the complex with 1:1 stoichiometry based on the standard definition of the stability constant (eq 3).

$$\Delta\delta_{obs} = \frac{\Delta\delta_C}{2[GA]_0}\left\{[NF]_0 + [GA]_0 + \frac{1}{K_{11}} - \left(\left([NF]_0 + [GA]_0 + \frac{1}{K_{11}}\right)^2 - 4[NF]_0[GA]_0\right)^{1/2}\right\} \quad (4)$$

Here, $\Delta\delta_{obs}$ and $\Delta\delta_C$ are the observed variation of chemical shifts of the selected GA proton (δ 1.05 ppm) in the NMR spectra and its value for the fully complexed GA, respectively. On the basis of the Job's plot data (Figure 3), to estimate the stability constant we have assumed that NF forms a complex of 1:1 stoichiometry with the dimer of the GA molecule. Experimentally measured dependence of the NMR chemical shift of GA protons on NF concentration at a constant GA concentration assuming that NF forms a complex with the dimer of the GA molecule is shown in Figure 4. The resulting value of the stability constant, $K_{11} = 1.2 \times 10^5\ M^{-1}$, obtained from formula 4 suggests an extremely high stability of the complex. Of note, since formula 4 is valid only for 1:1 stoichiometry of the complex, and for the general case, similar calculations require a much higher level of effort, it should be emphasized that experimentally measured $\Delta\delta_{obs}$ is analyzed under the assumption that NF forms the 1:1 complex with the dimer of the GA molecule, i.e., the ratio of [NF]/[GA] = 1.0 in Figure 4 corresponds to 1:2 molar stoichiometry of the NF−GA complex. Also, dimer concentrations of GA were used in calculations of the stability constant using formula 4.

Note that such high values of the stability constants were also observed for the complexes of GA with a number of other drugs.[1,9,19] For comparison, the stability constants of cyclodextrin complexes, widely used in pharmacology, are on the average $10^3\ M^{-1}$, 2 orders of magnitude lower than the value observed here for GA.[20,21] It should be noted that all measurements of stability constants and the stoichiometry of a complex were performed for the model system in water−alcohol solutions rather than in a real physiological medium. This is primarily due to the extremely low solubility of the initial components in water which precludes the analysis of the concentration dependences of the NMR line shifts or the intensities in the UV−vis spectrum. Note that the experiments conducted in the water−alcohol solutions of cyclodextrins[20,21] have shown that the addition of small amounts of methanol renders no effect on the stoichiometry and only slightly decreases the stability constants of the inclusion complexes.

The estimation of the stability constant from the data of relaxation measurements (see Table 1) results in $K_{11} \approx 6 \times 10^4\ M^{-1}$ for 30% methanol solution and $K_{11} \approx 1 \times 10^3\ M^{-1}$ for pure methanol.

Influence of the Complexation on the Reactivity of Nifedipine. In our previous study, we have suggested that the interaction of NF with the binding site of the L-type calcium receptor could involve a single electron-transfer step from one of its amino acid residues, tyrosine, to the NF molecule.[5] It has been shown that this reaction results in the transformation of NF into its nonreactive nitroso form (NONF) and could play a key role in the dissociation of the ligand−receptor complex. With the use of another ion channel blocker, Lap, it has been shown that the enhancing influence of GA on the therapeutic activity of a drug might be associated with its impact on the efficiency of the single electron-transfer step involving a drug and the amino acids forming the receptor's binding site.[6,9] The CIDNP technique was used to demonstrate that in the complex of Lap with GA the efficiency of electron transfer from an amino acid has been substantially reduced. This also results in the drop of the product yield and, in turn, might prolong the therapeutic action of the drug.

In the present article, the same approach based on the CIDNP application[5,6] has been employed to study the influence of the complexation with GA on the electron-transfer efficiency from tyrosine to NF. Of note, tyrosine was selected as a partner in

TABLE 1: Relaxation Parameters of Methyl Protons of NF and the NF−GA Complex

solvent	[GA] (mM)	I_1	T_{21} (s)	I_2	T_{22} (s)
CD_3OD	0	1.00	1.42 ± 0.035	0	
CD_3OD	0.7	0.85 ± 0.07	0.97 ± 0.06	≤ 0.15	0.06 ± 0.02
30% CD_3OD/D_2O	0.7	≤ 0.09	0.74 ± 0.14	0.91 ± 0.09	0.07 ± 0.01

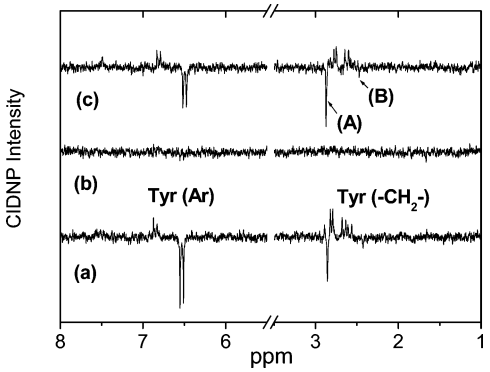


Figure 5. Aromatic and aliphatic parts of the time-resolved CIDNP (TR CIDNP) spectrum observed during irradiation (128 laser pulses, zero delay time) of the reaction mixture of nifedipine (NF) and tyrosine (Tyr) in D_2O/CD_3OD (4:1), in the presence and in the absence of GA: (a) 2 mM NF and 10 mM Tyr; (b) 2 mM NF, 10 mM Tyr, and 2 mM GA; (c) 2 mM NF and 10 mM Tyr with HCl added (pH 2.5). In previous studies (ref 5), lines A and B were attributed to the corresponding pyridinium cation and nitroso form of nifedipine (NONF), respectively.

the single electron-transfer step with NF, since it has been shown[7] that, in the L-type receptor binding site, Tyr is most closely located to the NF molecule which blocks the receptor. It was demonstrated that in this ligand−receptor complex, significant charge transfer from Tyr to NF is favorable,[7] thus facilitating the possible single electron-transfer step. Also, single electron transfer from Tyr to NF is stipulated by the beneficial relationship between the half-wave redox potentials of Tyr and NF ($E^{+}_{1/2}$(Tyr) = 0.9 V, $E^{-}_{1/2}$(NF) = −1.39 V).[5] For detection purposes, the photoinduced electron-transfer step in this experiment was initiated by the laser pulse at the absorption wavelength of NF (308 nm). The observation of CIDNP effects is an unambiguous evidence of the radical reaction mechanism, and the intensity of the CIDNP signal is generally proportional to the radical concentration in a sample. Time-resolved CIDNP spectra were taken at zero time point after the reaction, in order to separate the primary electron-transfer step and to exclude the contribution from the secondary radical processes in the bulk. The presaturation technique used for TR CIDNP allows us to suppress an equilibrium NMR spectrum, so only polarized groups of nuclei (in this case, protons) are observed in the resulting NMR spectra. Figure 5, parts a and b, shows CIDNP spectra taken during the irradiation of the NF and tyrosine mixture in the presence and in the absence of GA. The polarized signals from aromatic and aliphatic protons of tyrosine as well as polarizations from the products of transformation of the NF radical anion appear in the spectra (Figure 5) as a set of enhanced absorption and emission lines. Previous studies[5] of the single electron-transfer step in this process in the absence of GA based on comprehensive interpretation of TR CIDNP spectra has allowed us to suggest the detailed mechanism of the reaction between NF and tyrosine. A 5-fold excess of Tyr with respect to NF (10 mM Tyr and 2 mM NF) was used to suppress the direct photodecomposition of NF[5] which is capable of hampering the analysis of CIDNP effects generated through the single electron-transfer step. Of special importance is the complete disappearance of polarized signals when 2 mM GA solution is added to the reaction mixture (Figure 5b). It is suggested that this observation is due to the formation of the NF complex with GA that completely blocks the electron transfer between NF and tyrosine. To exclude possible effects of the changes in the acidity of the solution (GA is known to be a weak acid), the experiments in the absence of GA were also carried out with the addition of hydrochloric acid up to pH 2.5. Figure 5c shows that the intensity of polarization does not change with the decrease of pH.

Conclusion

^{1}H NMR relaxation measurements and UV−vis study have produced illustrative evidence that NF forms stable complexes with GA within a wide concentration range, from 0.05 to 5 mM. This leads to considerable increase in water solubility of NF. At low GA concentrations (below 1 mM), NF forms an inclusion complex where each NF molecule is bound by two molecules of GA. At higher concentrations, GA forms large micelle-like aggregates which further increase the water solubility of NF.

Formation of the complex with GA significantly alters the reactivity of NF. Nuclear polarization effects generated through single electron transfer from NF to tyrosine disappear if NF is bound in the inclusion complex with GA. It is reasonable to conclude that NF in the inclusion complex with GA is incapable of participating in the single electron-transfer step with an amino acid residue which is in close contact with NF when it is bound in the binding site of the L-type calcium receptor. This, in turn, means that, in the complex with GA, NF could more efficiently block the receptor.

This result has evident practical implications. Taking into account recent discoveries of the increased therapeutic activity of GA complexes with various biologically active compounds, our result allows us not only to suggest the decrease of the therapeutic dose of an active drug due to complexation but also to regulate the lower limit of the therapeutic action of a drug using different concentrations of GA. This is of special importance in the case of highly toxic drugs. In conclusion, it is worth noting that the predictive capability of the employed physicochemical research approaches allows one to significantly reduce the scope of animal studies.

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