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Association studies of aggregated aqueous lutein diphosphate with human serum albumin and α_1-acid glycoprotein in vitro: Evidence from circular dichroism and electronic absorption spectroscopy

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Abstract—Water-dispersible C40 carotenoid derivatives, with increased utility in mammalian therapeutic applications, include natural stereoisomer-based (3*R*,3′*R*,6′*R*)-lutein (β,ε-carotene-3,3′-diol) derivatives. Esterification with inorganic phosphate and conversion to the sodium salt produced compounds (lutein diphosphate sodium salt; 'LdP') capable of forming red-orange aqueous suspensions after addition to USP-purified water. The aqueous dispersibility of this diphosphate salt reached 29 mg/mL without the addition of heat, detergents, co-solvents, or other additives, and was a potent direct scavenger of superoxide anion (by EPR spectroscopy) in an isolated human neutrophil assay. In the current study, preliminary evidence of the aqueous aggregation of this compound in EPR studies was confirmed using circular dichroism (CD) and electronic absorption (UV–vis) spectroscopy. Evidence for H-type ('card-pack') and J-type ('head-to-tail') self-assemblies was obtained. In vitro analysis of the potential binding interaction between LdP and human serum albumin (HSA) and α_1-acid glycoprotein (AGP) revealed only non-specific binding with HSA (and none with AGP), contrasting with previous reports of direct interaction between astaxanthin-based soft drugs and the major plasma protein albumin. The rapid in vivo cleavage of this phosphodiester by promiscuous mammalian phosphatases may overcome the aqueous aggregation of the formulated compound. This difference in potential plasma protein interaction with prior reports reflects the subtle structural differences inherent in either the parent carotenoid scaffolds and/or the esterifying moieties.

Recently, the synthesis (using principles of retrometabolic drug design) of a novel lutein-based diphosphate soft drug was described (Fig. 1).[1] This compound exhibited significant water-dispersibility, ~29 mg/mL in USP-purified water. The compound was a potent direct scavenger of superoxide anion in an isolated human neutrophil assay, effective at millimolar (mM) concentration. Apparent aggregation of LdP was observed during radical scavenging studies using an electron paramagnetic resonance (EPR) spectroscopic assay. The preclinical evaluation of LdP was furthered by analyzing its plasma protein binding characteristics, described herein.

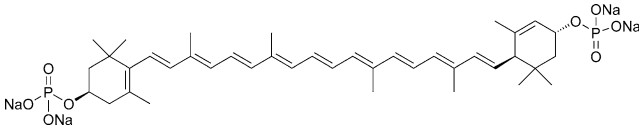

Figure 1. Chemical structure of lutein diphosphate sodium salt utilized in current spectroscopic investigations.

In the current study, the potential binding of lutein diphosphate sodium salt ('LdP') to human serum albumin (HSA) and the acute-phase protein α_1-acid glycoprotein (AGP) was studied using circular dichroism (CD) and electronic absorption (UV–vis) spectroscopy. Evidence for both H-type ('card-pack') and J-type ('head-to-tail') self-assembly[2] was obtained in Ringer buffer solution. Only non-specific binding to HSA was identified, which increased the relative proportion of head-to-tail aggregates in aqueous Ringer buffer solution. No interaction with AGP was identified. The

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water dispersibility of this compound, in part enabled by aqueous supramolecular assembly, appeared to be only minimally modified in the presence of these major plasma proteins. Therefore, the rapid in vivo cleavage of this phosphodiester—as has been reported for other phosphate and phosphoryloxymethyl (POM) pro-drugs[3–7]—may be important in generating free lutein for lipoprotein binding after parenteral administration.[8]

Essentially fatty acid-free HSA and human serum AGP were obtained from Sigma and used as supplied. Double-distilled water and spectroscopy grade dimethylsulfoxide (DMSO, Scharlau Chemie S.A., Barcelona, Spain) and ethanol (EtOH; Chemolab, Budapest, Hungary) were used. Natural source lutein (3*R*,3′*R*,6′*R*-β,ε-carotene-3,3′-diol) was used as supplied (Chromadex, Santa Ana, CA). Lutein diphosphate sodium salt ('LdP'; *R*,*R*,*R*-stereoisomer) was synthesized as recently described[1] and used in all spectroscopic investigations (purity >90% by HPLC, as AUC).

Preparation of lutein diphosphate sodium salt stock solution. A pre-weighed amount of LdP was dissolved in a small volume of DMSO. The carotenoid concentration was determined by measuring the optical density of the stock solution diluted by EtOH in a 1 cm cuvette at 447.5 nm (in EtOH the ε_{max} of lutein is 145,100 M^{-1} cm^{-1}).[9]

Circular dichroism (CD) and UV–vis electronic absorption spectroscopy measurements. CD and UV–vis spectra were recorded on a Jasco J-715 spectropolarimeter at 37 ± 0.2 °C in a rectangular cuvette with 1 cm pathlength. Temperature control was provided by a Peltier thermostat equipped with magnetic stirring. All spectra were accumulated three times with a bandwidth of 1.0 nm and a resolution of 0.5 nm at a scan speed of 100 nm/min. CD spectra were recorded and displayed as 'Θ' (ellipticity) in units of millidegrees (mdeg). Difference CD spectra were obtained by subtracting the CD spectrum of the proteins alone from those of the mixtures of LdP–HSA and LdP–AGP, respectively.

CD–UV–vis titration of serum proteins with LdP in pH 7.4 Ringer buffer solution at 37 °C. Protein solution, 1.8 mL ([HSA] = 185 μM and [AGP] = 29 μM) was placed in the cuvette with 1 cm optical pathlength, and small amounts of the ligand stock solution were added with an automatic pipette in microliter (μl) aliquots. The CD and absorption spectra were recorded at different ligand/protein (*L*/*P*) molar ratios. DMSO added with the ligand never exceeded 5% (v/v).

UV–vis and CD spectral properties of LdP in organic and aqueous Ringer buffer solutions. The visible absorption spectrum of LdP measured in ethanolic solution was typical for all-*trans* carotenoids having β,ε end-groups (Fig. 2): the strong absorption band between 350 and 550 nm exhibited vibrational fine structure, and the peak maxima were nearly equivalent with that of the purified natural source lutein used as synthetic starting material [(all-*E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol]. Therefore, as expected, chemical derivatization of the different end-groups did not affect the light absorption properties of the polyene chromophore. The solvent-induced bathochromic (red) shift is characteristic for carotenoid compounds, and could be used to test for the presence of the intact polyenic moiety. Upon increasing the solvent polarizability, the main absorption peaks shift to higher wavelengths.[10] The absorption curve of LdP recorded in the more polarizable solvent dimethylsulfoxide (DMSO) clearly followed this rule, exhibiting a 13–14 nm red shift in relation to the ethanolic spectrum (Fig. 2). It is difficult to measure the CD activity of true molecular solutions of chiral carotenoids above 350 nm at room temperature due to the weak chiral perturbation of the terminal stereocenters on the polyene π–π* electronic transition.[11] Phosphor, bound via oxygen to the β-ionone ring, is not expected to influence the CD pattern[12] and accordingly no definite CD band was observed either in the ethanolic or DMSO solutions of LdP (Fig. 2). Below 350 nm, however, the CD curve of LdP was similar to the parent *R*,*R*,*R*-lutein molecule (data not shown), indicating the intact stereochemistry of the β,ε-end-groups in the synthetic derivative.

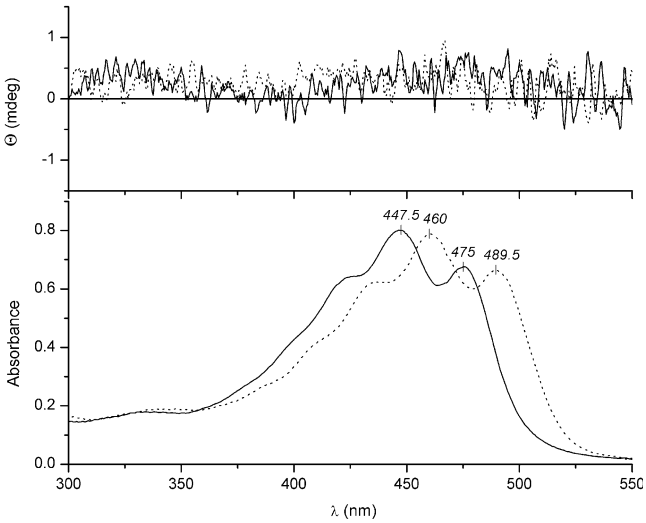

Figure 2. CD and vis spectra of lutein diphosphate sodium salt (LdP) in EtOH (solid line) and in DMSO (dotted line) at 25 °C (c = 5.52 μM). The λ_{max} at 447.5 nm agrees well with literature values for natural (*R*,*R*,*R*) all-*trans* lutein in organic solvent.[18]

The main absorption band of LdP in Ringer buffer solution exhibited a large hypsochromic (blue) shift (448–398 nm), loss of vibrational structure, bandwidth narrowing, and hypochromism—all indicating the formation of 'card-pack' (or H-type) aggregates in this solution (Fig. 3).[13,14] The absorption curve is mainly determined by the light absorption properties of 'card-pack' aggregates, although the long-wavelength tail and the shoulder around 500 nm suggested the simultaneous presence of 'head-to-tail' (J-type) carotenoid assemblies.[15]

The long-wavelength-negative and short-wavelength-positive Cotton effects (CE) centered around 417 and 374 nm, respectively (Fig. 3) demonstrated the left-handed (M) organization of the LdP molecules in-

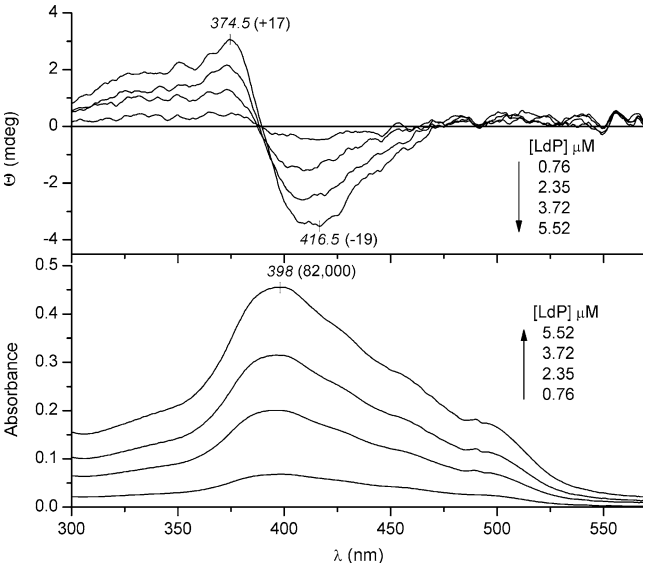

Figure 3. CD and vis spectra of LdP in Ringer buffer solution at different carotenoid concentrations (pH 7.4, $t = 37$ °C). The λ_{max} is blue-shifted relative to the solutions of LdP in organic solvents (cf. Fig. 2), and shows loss of vibrational fine structure, bandwidth narrowing, and hypochromism—consistent with the formation of 'card-pack' aggregation.

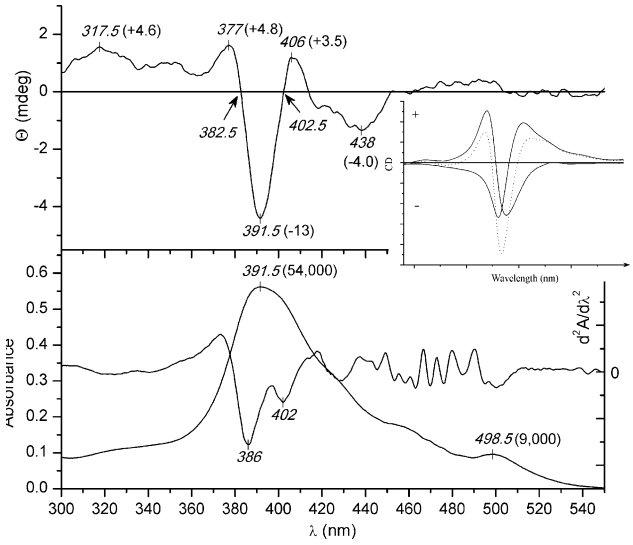

Figure 4. CD and vis spectrum and its second derivative curve (right axis) of LdP dissolved in double-distilled water ($c = 10$ μM, $t = 25$ °C). In parentheses, values of the molar absorption (ε) and the molar circular dichroic absorption ($\Delta\varepsilon$) coefficients of resolved peaks are indicated. Inset: illustration of the arithmetical sum of two hypothetical, right-handed (higher wavelengths) and left-handed (lower wavelengths) exciton CD band pairs (solid lines) resulting in a polyphasic CD curve (dotted line) similar to that obtained experimentally.

side the 'card-pack' assemblies.[13] No net CD activity was observed in the long-wavelength region of the spectrum (above 450 nm). It should be noted that the parent lutein molecules also form left-handed 'card-pack' aggregates in aqueous organic solvents; they display, however, much higher CD intensities.[13] Thus, the similarity between the CD band pattern of lutein and LdP aggregates suggested the role of the absolute configuration of the stereocenters in determination of the helicity of the arrangement of interacting carotenoid molecules, while the presence of the phosphate moieties appeared to alter the degree of the chiral packing of the polyene chains—but not the chirality of the intermolecular arrangement. This conclusion, however, is valid only when the negative charges of the phosphate groups are effectively shielded by the cations of the buffer solution; in double-distilled water 'card-pack' type self-assemblies of LdP molecules exhibit a qualitatively distinct CD motif from that measured in Ringer solution (Fig. 4). The appearance of polyphasic CD bands (positive–negative–positive) in the region of the blue-shifted absorption band indicates the co-existence of two [right- (at longer wavelengths) and left-handed (at shorter wavelengths)] 'card-pack' aggregates, of which the negative bands overlap and thus enhance each other (see the inset in Fig. 4). Due to the slight difference between the excitation energies of these assemblies, their UV peaks were not resolved in the absorption spectrum, but minima of the second derivative curve revealed their exact positions (Fig. 4). To explain the CD spectral changes observed in distilled water, it should be noted that right- (P) and left-handed (M) assemblies of a given chiral carotenoid molecule can be formed, but—as encoded by the absolute configuration of the asymmetric centers—the equilibrium between them is shifted toward the lower-energy form in the absence of any perturbing effect. In buffer solution where the negative phosphate charges were shielded and the Coulombic repulsion did not act, the left-handed organization appeared to be more stable. Due to the strong electrostatic interactions which arose in distilled water, the energy difference between the oppositely handed assemblies decreased, and both forms appeared in a nearly equal ratio, resulting in the observed CD curve (Fig. 4). It should be noted that these assemblies were not mirror images; their intermolecular overlay angles were opposite, but had no equal values. This was indicated by the different excitation energies (see the second derivative curve in Fig. 4), as the exciton coupling energy depends on the angle between the interacting chromophores.[15]

CD and UV–vis spectroscopic investigations of the interaction of LdP with human serum albumin (HSA) and human α_1-acid glycoprotein (AGP). The interaction of LdP with HSA was studied near the approximate physiologic serum concentration of the plasma protein (~0.6 mM) at low *L*/*P* ratios (Fig. 5). Despite the presence of the plasma protein in excess concentration relative to the carotenoid ligand, HSA was not able to prevent the aqueous aggregation of the carotenoid molecules; both the CD and absorption spectra suggest the formation and persistence of 'card-pack' aggregates in the combined solution. The blue-shifted absorption peak could be seen as a shoulder around 385 nm. The excitonic CD band pair exhibited the same signs and positions as those measured in buffer solution alone (cf. Fig. 3), indicating that the albumin molecules did not alter the chirality of the 'card-pack' aggregates.

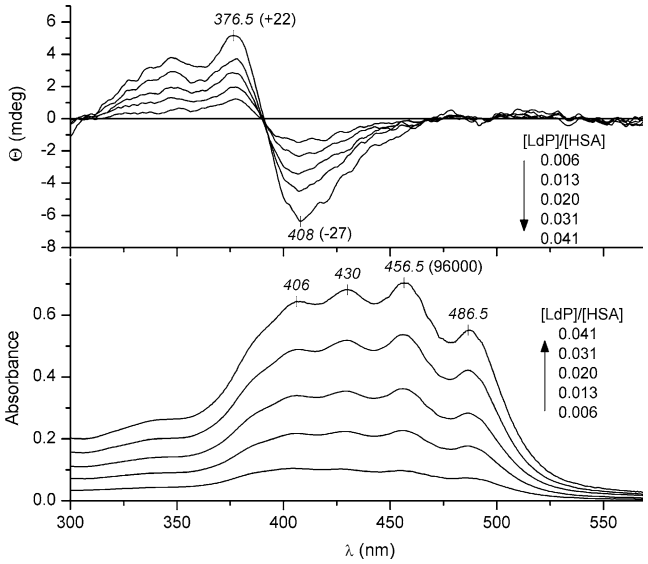

Figure 5. CD and vis spectra of LdP in the presence of fatty acid-free HSA at different *L*/*P* ratios (pH 7.4 Ringer buffer, $t = 37$ °C, [HSA] = 185 μM). In parentheses, values of the molar absorption (ε) and the molar circular dichroic absorption (Δε) coefficients of resolved peaks are indicated.

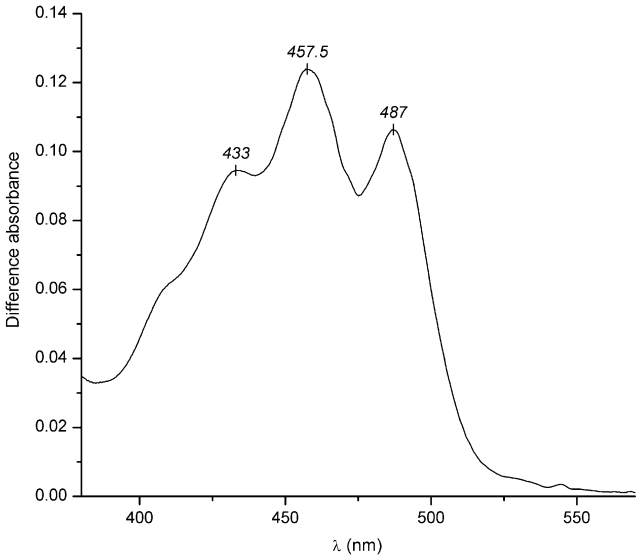

Figure 6. Difference electronic absorption spectrum of LdP (see text for details).

In comparison to the data obtained in Ringer buffer solution (Fig. 3), however, the visible absorption band showed that a significant fraction of LdP molecules did not participate in the formation of 'card-pack' assemblies (Fig. 5). The visible spectra displayed three vibrational peaks at 430, 456, and 486 nm, respectively, which, for the peaks at 456 and 486 nm, were bathochromically (red)-shifted by ~10 nm in relation to the corresponding peak positions measured in EtOH. To estimate the ratio of LdP molecules contributing to this spectral alteration, a simple calculation was made. The absorption spectrum of LdP measured in buffer solution alone at 2.35 μM (see the curve in Fig. 3) was substracted from that of the LdP–HSA mixture obtained at the same carotenoid concentration ($L/P = 0.013$, see the curve in Fig. 5). In comparison with the ethanolic visible spectrum of LdP measured at 5.52 μM, the maximum absorption intensity value of the resulting difference absorption spectrum (Fig. 6) was used to calculate the concentration of the 'non-card-pack' aggregated carotenoid molecules. The calculation yielded 0.85 μM, which is 36% of the total LdP concentration of the sample solution (2.35 μM, $L/P = 0.013$).

A comparison of the absorption curves of LdP–HSA mixtures with the UV–vis spectra of 'head-to-tail' (J-type) lutein diacetate assemblies previously reported,[15] and of the natural apocarotenoid bixin reported to bind to HSA in monomeric form,[16] suggested that there was no specific binding interaction between the LdP molecules and HSA in the current in vitro evaluation. Rather, a non-specific, weak superficial association occurred between the protein surface and the carotenoid assemblies, which resulted in an increase of the fraction of the 'head-to-tail' type aggregates, which lacked any definite chiral organization. This conclusion is strongly supported by the total absence of extrinsic CD activity in the spectral range of the vibrational sub-bands (Fig. 5), and contrasts with the evidence of association of astaxanthin conjugates (succinic acid salts and dilysine salts) with HSA in previous studies of plasma protein binding interactions.[13,14,17] Whether this is attributable to the different parent carotenoid scaffold (astaxanthin vs lutein) or physicochemical differences in the esterifying moieties (phosphates vs succinates/amino acids) remains unknown.

The CD and electronic absorption spectra measured in the presence of AGP again demonstrated the aqueous aggregation of the ligand molecules with no appreciable evidence of a carotenoid–AGP binding interaction (Fig. 7).

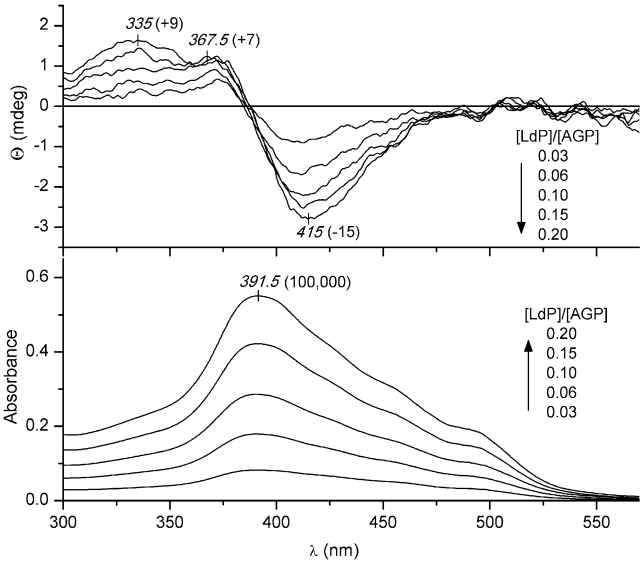

Figure 7. CD and vis spectra of LdP in the presence of human AGP at different *L*/*P* ratios (pH 7.4 Ringer buffer, $t = 37$ °C, [AGP] = 29 μM).

In Ringer buffer solution, LdP formed primarily H-type ('card-pack') aggregates, with evidence of a smaller percentage of J-type aggregates in this aqueous solution. The CD and UV–vis spectroscopic data suggested a non-specific binding interaction between LdP and HSA, which increased the relative percentage of J-type ('head-to-tail') aggregates in Ringer buffer solution. No evidence was seen for interaction with AGP. The lack of association of this lutein-based soft drug with the major plasma protein HSA, and the acute-phase protein AGP, suggests that subtle differences between the carotenoid scaffolds and/or esterifying moieties may be important for these interactions to take place. The rapid cleavage of phosphate esters in human serum for the aggregated carotenoids may overcome the lack of association with plasma proteins after parenteral administration in mammals.

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