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Circular dichroism and absorption spectroscopic data reveal binding of the natural *cis*-carotenoid bixin to human α_1-acid glycoprotein

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Abstract

Using circular dichroism (CD) and electronic absorption spectroscopy techniques, interaction of the natural dietary *cis*-carotenoid bixin with an important human plasma protein in vitro was demonstrated for the first time. The induced CD spectra of bixin obtained under physiological conditions (pH 7.4, 37 °C) revealed its binding to the serum acute-phase reactant α_1-acid glycoprotein (AGP), a member of the lipocalin protein family. Spectral features of the extrinsic Cotton effects of bixin suggested the inclusion of a single, chirally distorted ligand molecule into the asymmetric protein environment. Compared with the absorption spectra obtained in ethanol and benzene, the strong red shift of the main absorption peak of AGP-bound bixin indicated that the proposed binding site was rich in aromatic residues, and also suggested that hydrophobic interactions were involved in the binding. Using the data obtained from the CD titration experiments, the association constant ($K_a = 4.5 \times 10^5\ M^{-1}$) and stoichiometry of the binding (0.15) were calculated. The low value of the stoichiometry was attributed to the structural polymorphism of AGP. To the authors' knowledge, the current study

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represents the first human lipocalin protein for which carotenoid binding affinity has been explored in vitro with these techniques.

Keywords: Bixin; α_1-Acid glycoprotein; Circular dichroism spectroscopy; *cis*-Carotenoid; Induced chirality; Lipocalin; Red shift

1. Introduction

Annatto is an orange-red colorant obtained through extraction of the seeds of the tropical tree *Bixa orellana*. The principal coloring constituent of annatto is a *cis*-apocarotenoid known as bixin (Fig. 1). One end group of the asymmetric bixin molecule consists of a carboxyl group, and the other a carbomethoxy group. The hydrophobic central backbone consists of a rigid polyene chain with a *cis*-double bond situated near the methyl ester end of the molecule [1]. Bixin's use as a coloring agent and pigment extends into antiquity, and it is broadly used currently in the food industry as a coloring material [2–5]. Carotenoids such as β-carotene and lycopene are known to play an important role in human health as antioxidants, anti-inflammatory agents, as well as cancer chemopreventive agents [6,7]; however, little attention has been paid to the pharmacokinetics and pharmacological effects of bixin [8–11]. Protein–carotenoid interactions (for both natural and synthetic compounds) have been studied with multi-disciplinary techniques, as therapeutically active compounds often are carried by the significant plasma proteins such as human serum albumin (HSA). Ligand–protein interactions can deeply alter the pharmacologic fate and ultimate biological effects of potential drug-like molecules in the human body. However, the interaction of most of the >750 natural carotenoids with respect to plasma proteins and acute-phase reactants is an area that is largely unexplored.

It was recently reported that after dietary intake, bixin is present in human plasma [12]. Its associations with plasma proteins or lipoproteins after gastrointestinal absorption are unknown. Serum α_1-acid glycoprotein (AGP),[1] a member of the lipocalin protein family, is a highly heterogeneous, extensively glycosylated acute-phase glycoprotein ($M_w \approx 41{,}000$), consisting of a chain of 183 amino acids [13–15]. Under physiological conditions, the serum concentration of AGP is about 1 g/L. Its concentration increases several-fold following tissue injury, infection, presence of cancer, or other inflammatory stimulus that results in an acute-phase response. Despite extensive research, the precise biological function(s) of AGP is currently not well understood. Of the numerous potential biological roles attributed to AGP, the anti-inflammatory and immunomodulating roles appear to be the most important. Indeed, AGP has also been classified as a member of the immunocalin family, a lipocalin subfamily that modulates immune and inflammatory responses

[1] *Abbreviations used:* AGP, α_1-acid glycoprotein; CD, circular dichroism; CE, Cotton effect; L/P, ligand/protein molar ratio; mdeg, millidegree; UV/Vis, ultraviolet–visible.

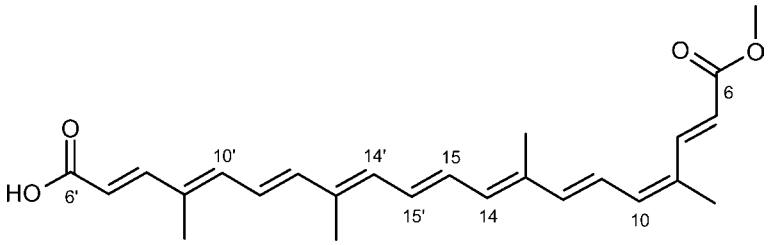

Fig. 1. Chemical structure of bixin (6,6′-diapo-ψ,ψ-carotenedioic acid monomethyl ester).

[14]. Several hundred basic, neutral, and acidic drugs have been reported to bind to AGP, underscoring its importance as a drug-binding transport protein [15]. At the present time, the X-ray crystallography structure of AGP has not been determined.

In the current study, the interaction between the naturally occuring *cis*-apocarotenoid bixin and AGP were studied in vitro under conditions of physiologic pH (7.4) and temperature (37 °C), utilizing circular dichroism (CD) and ultraviolet–visible (UV/Vis) spectroscopy. The spectroscopic data obtained strongly suggested the formation of non-covalent bixin–AGP complexes. The association constant and the stoichiometry of the binding were determined directly from the spectroscopic data. In addition, the chemical nature of the proposed binding microenvironment for the bixin ligand was characterized. The potential biological significance of the observed carotenoid–AGP interaction is subsequently briefly discussed.

2. Materials and methods

2.1. Materials

Human AGP purified from Cohn fraction VI (Catalog No. G-9885) was obtained from Sigma and used as supplied. According to the manufacturer, the purity of the AGP sample was 99% by agarose gel electrophoresis. The bixin sample was isolated from annatto (*B. orellana*) utilizing methods described previously [16]. Crystallization of bixin from glacial acetic acid yielded red crystals with a purity of 97% (HPLC). Double distilled water, HPLC grade ethanol (EtOH; Chemolab, Hungary), and spectroscopy-grade dimethyl sulfoxide (DMSO; Scharlau Chemie S.A., Barcelona, Spain) were used as supplied. All other chemicals were of analytical grade.

2.2. Preparation of AGP and bixin solutions

For spectroscopic measurements, solid AGP samples were weighed in a rectangular cuvette with 1 cm pathlength, and then dissolved in 2 ml of pH 7.4 Ringer buffer solution.The bixin stock solution was prepared in DMSO. The molar concentrations of AGP (1.3×10^{-4} M) and bixin (1×10^{-3} M) were calculated on the basis of their molecular masses of 41,000 and 394.5, respectively.

2.3. Circular dichroism and UV/Vis absorption spectroscopy

Circular dichroism and UV/Vis spectra were recorded between 300 and 600 nm on a Jasco J-715 spectropolarimeter at 25 ± 0.2 and 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. Induced CD is defined as the CD of the bixin–AGP mixture minus the CD of the AGP protein alone at the same wavelengths, and is expressed as ellipticity in millidegrees (mdeg).

2.4. Circular dichroism–UV/Vis titration of AGP with bixin in pH 7.4 Ringer buffer solution at 37 °C

Two milliliters of 1.3×10^{-4} M AGP protein solution was placed in a rectangular cuvette with 1 cm optical pathlength, and the ligand stock solution was added with an automatic pipette in 3 μl aliquots to achieve L/P molar ratios from 0.01 to 0.40. DMSO added with the ligand in no instance exceeded 5% (v/v) in the resulting solution.

3. Results and discussion

3.1. UV/Vis absorption spectra of bixin in organic and in aqueous solutions

The conjugated double bond system constitutes the light-absorbing chromophore of bixin, resulting in the strong absorption band above 400 nm (Fig. 2). This transition is electronic-dipole allowed, and it is oriented along the long axis of the molecule. The next higher energy transition occurs between 320 and 380 nm; in all-*trans*-carotenoids this gives rise to no net dipole moment, and thus no absorption band. However, the presence of one or more *cis*-double bonds may result in a dipole moment for this transition, and the so-called *cis*-peak appears in the spectrum of these *cis*-carotenoids, for which the energy and intensity depend on the position and number of the *cis*-bonds [17]. In benzene, the *cis*-peak of bixin appears at 364 nm with a molar absorption coefficient (ε) of 12,400 M^{-1} cm^{-1} (Fig. 2). Comparison of the UV/Vis spectra of bixin obtained in EtOH and benzene reveals pronounced solvent dependence, a feature characteristic to the electronic absorption spectra of polyene compounds [18]. Via favorable dispersion interactions, the increasing polarizability of solvent molecules decreases π–π* excitation energy of the polyene chromophore, which leads to a bathochromic shift of the UV/Vis absorption peaks. Since the polarizability of the aromatic π cloud of benzene molecules is higher than that of the bonding and non-bonding electrons of ethanol, the main absorption band is shifted by 10 nm to longer wavelengths in benzene (Fig. 2). An additional difference between these spectra is the broadening of the absorption band measured in EtOH, which also carries less pronounced vibrational fine structure than the peak obtained in benzene (the bandwidths, measured at half of

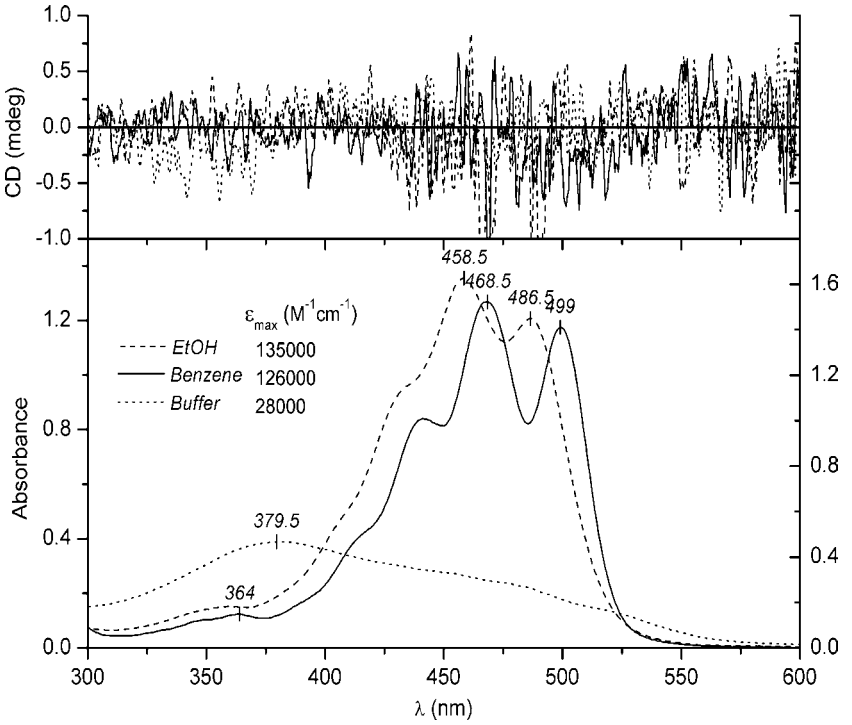

Fig. 2. UV/Vis and CD spectra of bixin in benzene (25 °C, 1.0×10^{-5} M), ethanol (25 °C, 1.2×10^{-5} M), and pH 7.4 Ringer buffer (37 °C, 1.4×10^{-5} M); ε values were calculated using the molar concentration of dissolved bixin.

the absorption maxima, are 3705 and 3937 cm^{-1} in benzene and EtOH, respectively). These spectral observations emphasize the marked influence of the immediate solvent microenvironment on the spectroscopic behavior of the carotenoid chromophore. In this case, the EtOH molecules establish stronger intermolecular interactions with bixin than do the benzene molecules, i.e., intermolecular H-bonds and dipole–dipole interactions with the heteroatomic end groups of bixin [19,20]. Since both the ester and carboxyl groups are conjugated with the π-electron system, these interactions alter the electronic as well as the vibrational transitions of the polyene chain leading to the changes in the UV/Vis spectrum described above.

Despite the presence of an ionizable carboxyl group, bixin shows very poor aqueous solubility due to its predominantly hydrophobic polyenic constitution. Its UV/Vis spectrum recorded in pH 7.4 Ringer buffer is quite distinct from those obtained in organic solvents; the principal absorption peak is strongly blue-shifted and shows large hypochromism (Fig. 2). Only a weak, residual absorption was measured in the visible range, including a slightly red-shifted tail. These changes can be attributed to the self-association of bixin molecules; the blue-shifted peak is the consequence of card-pack (H-type) aggregation of the polyene chains, while the weak

bathochromic absorption suggests the simultaneous presence of the so-called J-type assemblies [21].

Due to a lack of intrinsic chiral elements, bixin exhibits no CD activity either in organic or aqueous solvents (Fig. 2).

3.2. Absorption and CD spectral properties of the bixin–AGP mixture

Circular dichroism and UV/Vis titration experiments were performed by repeated addition of small aliquots of the bixin stock solution to AGP dissolved in pH 7.4 Ringer buffer at 37 °C. Upon addition of bixin, three extrinsic Cotton effects (CE) appeared in the visible as well as in the UV region (Fig. 3). The wavelength position and shape of the lowest energy negative CD band nearly matched with the principal absorption peak, although its vibrational fine structure was blurred. A positive CE was measured in the *cis*-peak region of bixin centered at 363 nm. The intrinsic light absorption and CD activity of AGP did not allow spectroscopic measurements below 300 nm where a third, negative extrinsic CD band was also present. These induced CEs strongly suggest the binding of bixin to AGP. It is reasonable to postulate that inclusion of the CD-inactive bixin molecule into the chiral environment of AGP was the source of the extrinsic CD spectrum. The CD data suggested that those bixin molecules which were responsible for the induced CEs bound to the protein in monomeric form, and that no further ligand was bound in their immediate vicinity, i.e., no spectroscopic sign(s) of intermolecular exciton coupling was observed. The fact that the shapes and spectral positions of the fairly intense induced CD bands matched with the UV/Vis absorption peaks indicated that the asymmetric binding site forced the bound ligand molecules to adopt a chiral conformation. We suggest that the slightly shorter and longer portions of the bixin molecule (as measured from the *cis*-bond; Fig. 1) are slightly twisted around the *cis*-double bond, thus generating a CD-active chiral chromophore. Interestingly, the typical lipocalin protein bovine β-lactoglobulin showed no bixin binding ability at all (data not shown). Since both AGP and β-lactoglobulin belong to the same protein superfamily, this finding refers to the need of specific structural conditions for the protein binding of bixin.

The behavior of the UV/Vis absorption spectrum of bixin also supported the ligand/protein complex formation, and gave further insight into the binding mechanism. Similar to the spectrum obtained in EtOH, the vibrational structure of the main absorption peak of AGP-bound bixin was less pronounced (Fig. 3), and was even broader than that measured in EtOH (4171 cm^{-1} vs. 3937 cm^{-1}, L/P = 0.05). This may be the result of intermolecular H-bonds established between the binding site residues and the ligand molecule. Notably, the wavelength position of the visible absorption band was also altered, exhibiting a large bathochromic shift for the AGP-bound state (Fig. 3, cf. Fig. 2). The absorption maximum of the carotenoprotein complex was even at longer wavelength (474 nm) than that for bixin alone measured in benzene (468.5 nm), suggesting the binding site of bixin to be rich in aromatic residues. As has been demonstrated, aromatic side chains play an important role in the fixation of carotenoids in hydrophobic protein regions, essentially forming an an-

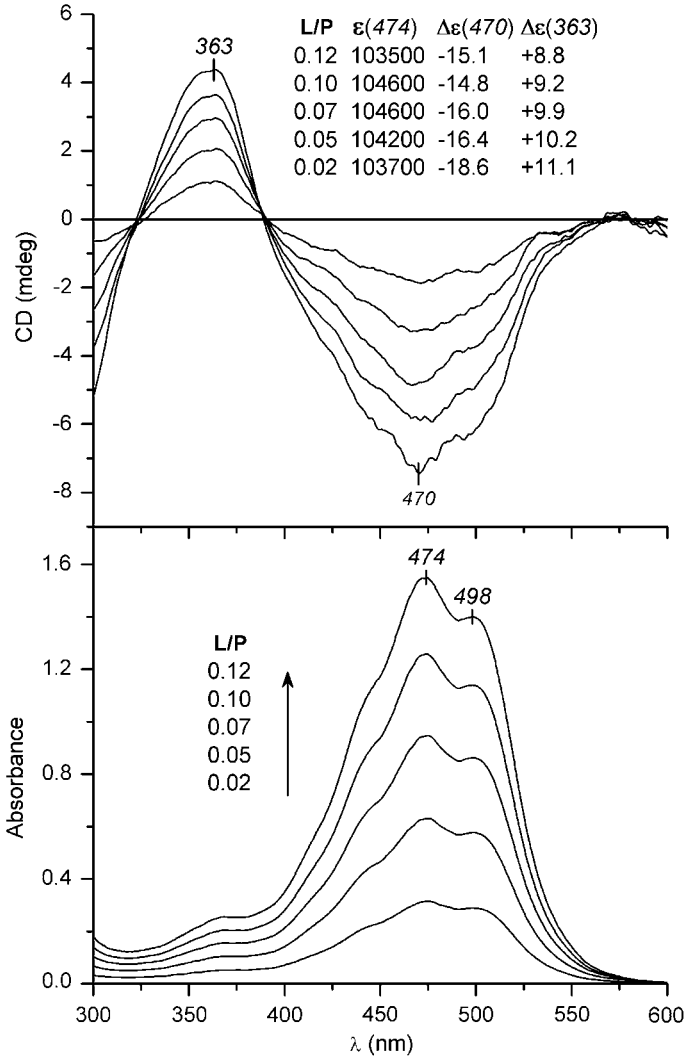

Fig. 3. Some representative induced circular dichroism and UV/Vis spectra obtained during titration of AGP with bixin in pH 7.4 Ringer buffer solution (cell length 1 cm; $c_{AGP} = 1.2 \times 10^{-4}$ M, $t = 37$ °C). Values of ligand/protein (L/P) ratios, molar absorption (ε), and circular dichroic absorption coefficients ($\Delta\varepsilon$) are indicated.

chor site for the chromophore [22,23]. Intermolecular π–π stacking between the carotenoid polyene chain and the π-electron system of the surrounding aromatic residues is responsible for the bathochromic shifts observed in these carotenoprotein complexes [23–25]. Investigation of the primary amino acid (AA) sequence of AGP reveals that the most aromatic residues are concentrated within the AA sequence 25–65 (Fig. 4). Interestingly, a 3D-molecular model of AGP constructed

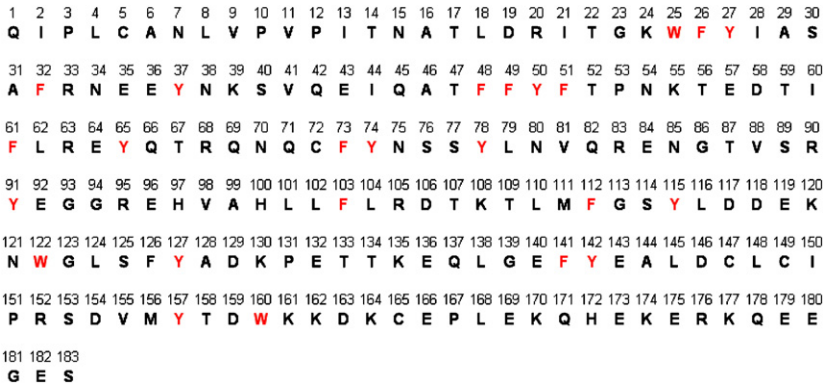

Fig. 4. Primary amino acid sequence of the ''A'' genetic variant of AGP (aromatic residues are highlighted by red).

by multiple sequence alignment showed that some of these aromatic residues participated in the formation of the wall and ''mouth'' of a central hydrophobic pocket thought to be the principal binding site of ligand molecules [26]. Additionally, Phe98, Tyr127, and Trp122 also appeared to contribute structurally to the binding cavity. The remaining aromatic residues were scattered throughout the surface of AGP or were buried within the protein matrix.

To obtain quantitative data on the bixin–AGP interactions, CD titration measurements were performed by adding bixin to Ringer buffer in which the AGP concentration was kept at a constant value. Amplitudes of the near-UV induced CD band at 363 nm were obtained, as the ellipticity/optical density ratio was the most favorable in that region. The CD values showed saturation after reaching the L/P ratio of 0.30 (Fig. 5), and the stoichiometry of the binding calculated from the titration curve was found to be 0.15 (Fig. 5). This suggested that only 15% of all AGP molecules in solution bound bixin molecules, as detected by CD spectroscopy. Therefore, the possibility of the existence of additional AGP binding sites, for which carotenoid binding gave rise to weaker induced CD signals (masked by the stronger bands of the previously described CD-active species), had to be taken into account. To address this question, a second UV/Vis titration experiment was performed, changing the L/P ratio from 0.13 to 1.61. If AGP were to bind bixin molecules in a ''CD-silent'' manner, then these molecules would also be prevented from participating in aqueous aggregation/supramolecular assembly (see Fig. 2). Thus, the UV/Vis spectra should show no alterations upon increasing the [bixin]/[AGP] ratio above 0.30, where saturation of the CD amplitudes was previously observed. However, the experimental spectral curves obtained at these L/P ratios did not support this supposition.

In Fig. 6, the quotients of the absorbance values measured at the visible maximum and at 365 nm are plotted against the L/P ratios. The quotients should be constant until the depletion of the bixin-binding capacity of AGP is reached; subsequently, the aggregation of unbound carotenoid molecules would result in a new absorption band between 300 and 400 nm (see Fig. 2). The quotient started to decrease begin-

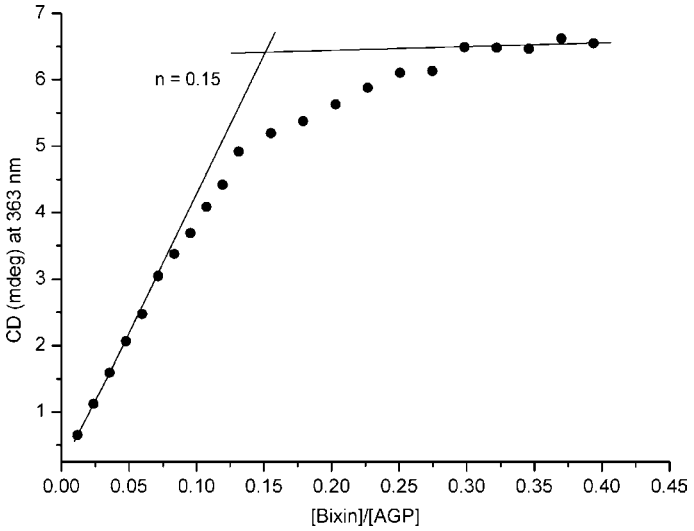

Fig. 5. CD data points obtained by titration of AGP with bixin in pH 7.4 Ringer buffer ($[AGP] = 1.2 \times 10^{-4}$ M, optical pathlength = 1 cm, $t = 37$ °C). Intersection of the lines fitted on the beginning and final linear parts of the titration curve gives the number of binding sites.

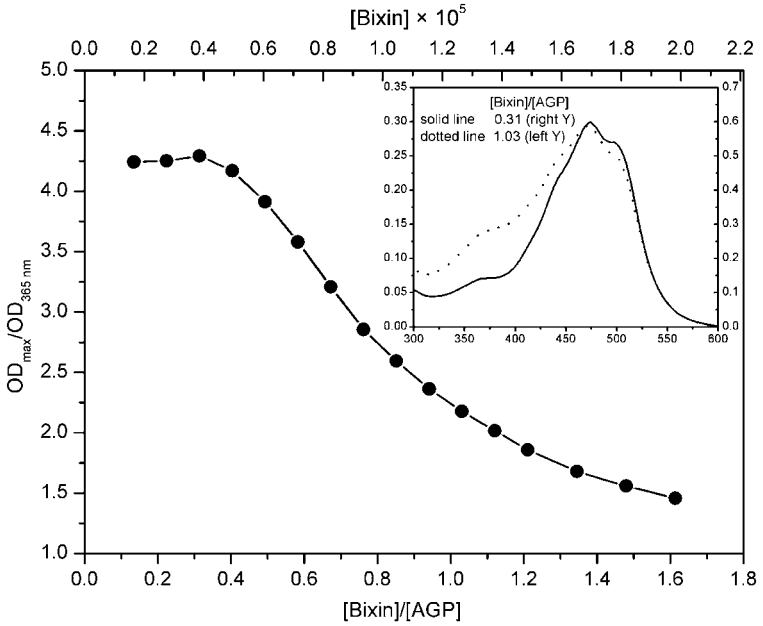

Fig. 6. Ratios of the maximum optical density (OD_{max}) of the visible absorption peak of bixin and the absorbance values measured at 365 nm ($OD_{365\ nm}$) plotted against [bixin]/[AGP] ratios and bixin concentrations, respectively (pH 7.4 Ringer buffer, $[AGP] = 1.2 \times 10^{-5}$ M, $t = 37$ °C). Inset shows absorption curves of bixin at different L/P values. Solid line: fully protein-bound state of bixin. Dotted line: the AGP binding site is saturated, the unbound carotenoid molecules form aggregates.

ning with the L/P value of 0.40, indicating saturation of the binding capacity of AGP and the start of the aqueous aggregation of bixin. Similar to the previous case, magnitude and shape of the induced CD bands remained unaltered after raising the L/P value above 0.30 (data not shown). Consequently, only a negligible fraction of AGP-bound bixin demonstrated no induced CD activity, and therefore we concluded that the CD titration data represented the entire binding process.

Assuming that all bixin molecules were bound to AGP at low L/P ratios, the linear portion of the binding isotherm (Fig. 5) was utilized to calculate the protein-bound and unbound fractions of bixin at higher L/P values. A linear curve fitting procedure (not shown) was performed on the first four CD values (L/P = 0, 0.01, 0.02, and 0.04). This produced the following linear equation:

$$\mathrm{CD}_{363\mathrm{nm}}(\mathrm{mdeg}) = 0.01375 + 385412.37 \times [\mathrm{bixin}]_{\mathrm{bound}} \quad (r^2 = 0.998),$$

which was then used to obtain the data necessary for a Scatchard analysis. The Scatchard plot constructed utilizing this estimation is shown in Fig. 7. The derived value of the association constant (K_a) is 4.5 ($\pm$ 0.2) $\times 10^5$ M^{-1}.

A potential explanation for the low value of the stoichiometry of the bixin–AGP binding takes into account the genetic polymorphism of AGP [13,15]. Commercially obtained AGP samples contain three main genetic variants (F1, S, and A), for which the relative abundance of the three variants was reported to be approximately 44, 29, and 27%, respectively [27]. The F1–S (44 + 29%) and the A fractions differ from each other in their primary amino acid sequences (22 residues), and also in their ligand binding properties [15,27–29]. Structural AGP variation is further increased by the presence of distinct forms of di-, tri-, and tetra-antennary carbohydrate chains, as demonstrated by identification of seven glycoforms of AGP using capillary electro-

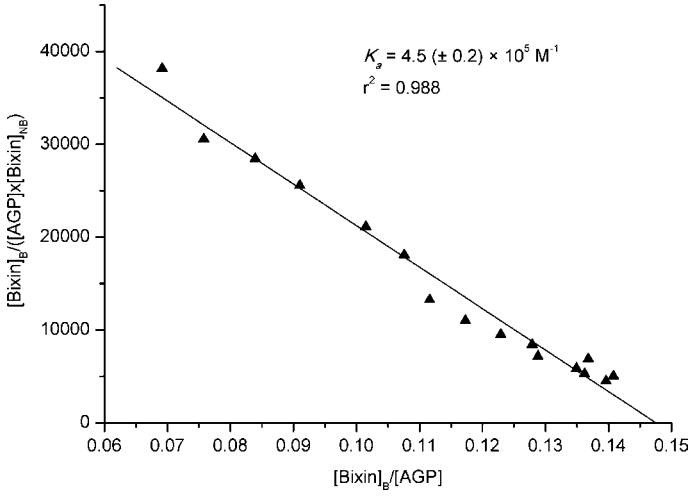

Fig. 7. Scatchard plot of monomeric binding of bixin to AGP. "B" and "NB" subscripts refer to the protein-bound and unbound fractions of bixin, respectively.

phoresis techniques [30]. The low stoichiometry of binding observed in the current study may therefore reflect the ability of only certain AGP structural variants to bind bixin, e.g., only variant A. Further study would be necessary to definitely identify those structural variants able to bind bixin.

4. Conclusion

The current study provides the first spectroscopic evidence for the in vitro binding of the natural dietary *cis*-apocarotenoid bixin to human α_1-acid glycoprotein under physiologic conditions of pH and temperature. Previous descriptions of the binding by lipocalins (such as the plant-derived violaxanthin deepoxidase/zeaxanthin epoxidase [31] and the lobster shell crustacyanin [32]) of carotenoids exist; to the authors' knowledge, however, this is the first human lipocalin found to exhibit binding affinity towards one of this highly important group of natural antioxidant, anti-inflammatory compounds. The spectral absorption data suggested that the immediate microenvironment of AGP-bound bixin was rich in aromatic residues, the spatial distribution of which appeared to be consistent with the main hydrophobic binding pocket of the protein. The low value of the stoichiometry of the bixin–AGP binding (0.15) further suggested that the structural variants of commercially available AGP might differ in their ability to bind bixin. It has recently been reported that the original β-sheet-rich structure of AGP changes to an α-helix-rich structure upon its binding to phospholipid membranes, with a concomitant decrease of ligand binding capacity [34]. Since AGP molecules have been detected on the surface of various human cells [13–15], this mechanism may play a role in the targeted delivery of bixin (and potentially other carotenoids) in vivo to effector cells involved in inflammation and/or immunological reactions. As the concentration of AGP is significantly increased both in serum and tissues in several diseases accompanied by free radical generation, the binding and transport of the poorly water-soluble antioxidant bixin by AGP might contribute to its beneficial biological activity in target tissue, e.g., protection against lipid peroxidation [10,33].

Acknowledgments

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