

Fourier-Transform Infrared Spectroscopic Studies on Avidin Secondary Structure and Complexation with Biotin and Biotin-Lipid Assemblies

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ABSTRACT Fourier-transform infrared studies have been carried out to investigate the secondary structure and thermal stability of hen egg white avidin and its complexes with biotin and with a biotinylated lipid derivative, *N*-biotinyl dimyristoyl phosphatidylethanolamine (DMBPE) in aqueous dispersion. Analysis of the amide I stretching band of avidin yielded a secondary structural content composed of approximately 66% β -sheet and extended structures, with the remainder being attributed to disordered structure and β -turns. Binding of biotin or specific association with the biotinylated lipid DMBPE did not result in any appreciable changes in the secondary structure content of the protein, but a change in hydrogen bond stability of the β -sheet or extended chain regions was indicated. The latter effect was enhanced by surface interactions in the case of the biotin-lipid assemblies, as was demonstrated by electrostatic binding to a nonspecific negatively charged lipid. Difference spectra of the bound biotin implicated a direct involvement of the ureido moiety in the ligand interaction that was consistent with hydrogen bonding to amino acid residues in the avidin protein. It was found that complexation with avidin leads to a decrease in bond length of the biotin ureido carbonyl group that is consistent with a reduction of sp^3 character of the C—O bond when it is hydrogen bonded to the protein. Studies of the temperature dependence of the spectra revealed that for avidin alone the secondary structure was unaltered up to $\sim 75^\circ\text{C}$, above which the protein undergoes a highly cooperative transition to an unfolded state with concomitant loss of ordered secondary structure. The complexes of avidin with both biotin and membrane-bound DMBPE lipid assemblies display a large increase in thermal stability compared with the native protein.

INTRODUCTION

Avidin is a homotetrameric protein present in the egg white of birds and reptiles (Green, 1975; Elo and Korpela, 1984). The molecular mass of the tetramer is 68 kDa, and the primary sequence of the 128-residue monomer is known (DeLange and Huang, 1971). Because of the extraordinarily high affinity ($K_d = 10^{-15}$ M) of avidin for the vitamin biotin and its derivatives, the avidin-biotin system is used extensively in various areas of biology and biotechnology (for reviews, see Bayer and Wilchek, 1980; Wilchek and Bayer, 1989, 1990).

In some of the applications of the avidin-biotin system, biotinylated lipids in which the biotin moiety is attached to the headgroup of a phospholipid such as phosphatidylethanolamine or phosphatidylserine are used. Such applications include localization of liposomes in studies on cell-liposome interaction (Bayer et al., 1979), noncovalent attachment of antibodies to cells (Loughrey et al., 1987),

and targeting of drug-loaded liposomes to tumor cells (Urdal and Hakomori, 1980; Rivnay et al., 1987). The biotinylated lipids have also found applications in the formation of two-dimensional crystals of proteins in monolayers that are suitable for solving the three-dimensional structure of these proteins and could prove very useful in materials research, particularly in developing biosensors (Ahlers et al., 1989; Kornberg and Darst, 1991). In addition, the specific interaction of avidin with the headgroups of biotinylated lipids in aqueous dispersion can be used as a model system for studying molecular recognition at cell surfaces. Some progress in this direction has already been made (Swamy and Marsh, 1993). The present study goes further in characterizing the structure of the lipid-bound protein and the molecular details of the interaction with the specific ligand.

In view of the wide applications of avidin-biotin systems in biology and of their relevance as a model system for studying surface recognition processes, it is important to characterize the structure of avidin and the effect of binding biotin as well as its membrane-bound conjugates. Equally of importance is to characterize in detail the molecular interactions that are involved in the recognition process. Studies of the interaction with the lipid conjugates of biotin are particularly necessary because protein binding to lipids could result in changes in the lipid membrane phase behavior and polymorphism as well as in alterations in the protein conformation and stability (see, e.g., Heimbürg et al., 1991; Heimbürg and Marsh, 1993). In previous studies we characterized the phases formed by a homologous series of

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Abbreviations: biotin-PE, diacyl *N*-biotinyl phosphatidylethanolamine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DMBPE, 1,2-dimyristoyl-*sn*-glycero-3-(*N*-biotinyl)-phosphoethanolamine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; FTIR, Fourier transform infrared spectroscopy; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid.

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diacyl *N*-biotinyl phosphatidylethanolamines (biotin-PEs) by using differential scanning calorimetry, ^{31}P -NMR spectroscopy, small-angle x-ray diffraction, and spin-label electron spin resonance spectroscopy (Swamy and Marsh, 1994; Swamy et al., 1993, 1994) and investigated the effect of avidin binding on the lipid phase behavior and polymorphism and the lipid chain dynamics (Swamy and Marsh, 1993 and in preparation). These studies have shown that the biotin-PEs form various unusual lipid phases and that avidin binding results in a significant alteration in their phase properties.

To determine the effects of binding of biotin and biotin-PEs on the structure and stability of avidin we have carried out Fourier-transform infrared (FTIR) studies of avidin and its complexes with biotin and with *N*-biotinyl dimyristoyl phosphatidylethanolamine (DMBPE) assemblies as a function of temperature. It was found that binding of both biotin and the biotin-lipid DMBPE does not significantly alter the secondary structure of avidin but enhances the thermal stability of the protein dramatically. FTIR difference spectroscopy is capable of revealing the molecular features of these interactions. In particular, it was found that complexation involves a shortening of the ureido carbonyl bond length in the biotin moiety.

MATERIALS AND METHODS

Materials

Avidin was purchased from Molecular Probes, Inc. (Eugene, OR). Biotin, biotin *N*-hydroxysuccinimide ester, and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO). D_2O was from Merck (Darmstadt, Germany). Dimyristoyl phosphatidylethanolamine (DMPE) was a product of Fluka (Buchs, Switzerland), and dimyristoyl phosphatidylglycerol (DMPG) was from Avanti Polar Lipids (Alabaster, AL). *N*-biotinylated phosphatidylethanolamine (DMBPE) was synthesized from DMPE and biotin-*N*-hydroxysuccinimide ester according to Bayer et al. (1979).

Sample preparation

Avidin was dissolved in 10 mM Hepes buffer, pH 7.4. This was lyophilized, and the resultant dry powder was redissolved in a volume of D_2O equal to that of the original solution. After another cycle of lyophilization followed by dissolving in D_2O , the protein was incubated at room temperature overnight to maximize H-D exchange. To prepare the avidin-biotin complex, D-biotin was added from a 100-mM stock solution in D_2O (solubilized and pH adjusted to 7.5 with 1 M NaOD/1 M DCl in D_2O) to an avidin solution, prepared in deuterated buffer as described above, to yield ~10% excess of biotin. This was incubated again for several hours at room temperature before the spectra were recorded. The complex of avidin with the biotinylated lipid (DMBPE) was prepared by mixing 0.15 μmol of the protein (tetramer) with 4 μmol of the lipid dispersed in deuterated buffer, followed by vigorous vortexing and incubation at room temperature for several hours.

FTIR spectroscopy

FTIR spectra were recorded in the range 400-4000 cm^{-1} at a nominal resolution of 4 cm^{-1} on a Bruker IFS 25 spectrometer. Samples were placed between two CaF_2 windows separated by a 50 μm -thick Teflon spacer. The spectrometer was flushed with dry nitrogen gas for at least 30

min before the spectra were recorded. For each spectrum 100 interferograms were recorded, summed, and apodized with a triangular function before Fourier transformation. The temperature of the sample was controlled to an accuracy of 0.1° by circulating thermostatted water through the metal housing supporting the sample cell. To ensure temperature equilibrium the sample was incubated at the desired temperature for at least 20 min before the spectra were recorded.

Fourier-transform self deconvolution was performed with software routines provided by the group of H. H. Mantsch, Canada (Kauppinen et al., 1981). Line narrowing of the unsmoothed spectra was performed with a triangular apodization function, assuming an initial bandwidth with a half-width at half-height of 17 cm^{-1} and a line-narrowing factor of $K = 1.5$. The components in the amide I region of the spectrum were fitted with a combination of Gaussian and Lorentzian bands by using the original spectrum, which was minimally smoothed to remove any water-vapor distortion without affecting the fundamental band shape. Band positions were determined from the Fourier self-deconvolution. Avidin-biotin (lipid) difference spectra were obtained by subtracting the original (smoothed) spectra of avidin or biotin lipid alone. The spectrum subtracted was scaled to correspond to the relative amount present in the composite spectrum from which it was subtracted. Temperature-dependent difference spectra were obtained from normalized, deconvoluted spectra (integrated area of 100 $\times \text{cm}^{-1}$). Normalization was performed by defining a linear baseline between the wave number limits of the subtraction. The parameter deduced from the difference spectrum was the integrated area of the positive region (i.e., above the base line); the total integral by definition is zero (see Heimburg and Marsh, 1993).

Concentration determination

The concentration of avidin was determined from its absorbance at 282 nm, using the extinction coefficient given by Green (1975). The concentrations of D-biotin and of DMBPE were determined gravimetrically.

RESULTS

Avidin secondary structure

The amide I, amide II, and lipid carbonyl regions in the FTIR spectra of avidin and its complex with D-biotin and with *N*-biotinyl dimyristoylphosphatidylethanolamine (DMBPE), recorded at 20°C in deuterated buffer, are shown in Fig. 1 A. The avidin spectrum exhibits a maximum at ~1633 cm^{-1} , which is the dominant feature of the amide I region for this protein, with shoulders at higher wave number. Although the major band at 1633 cm^{-1} is clearly indicative of a predominantly β -sheet and extended chain structure for the protein, other features of the secondary structure are not well resolved in the original spectrum. Comparison of the spectrum of avidin with the spectra of its complexes with biotin and with the biotin lipid DMBPE indicates only minor differences, suggesting that ligand binding involves little or no change in conformation of the protein at the secondary structural level.

To resolve the overlapping bands in the original spectra, band narrowing was performed by Fourier self-deconvolution. Conservative deconvolution reveals the presence of at least four bands in the amide I region of the avidin spectrum (Fig. 1 B). The positions of these bands were determined by band fitting to the deconvoluted spectrum. Then these band positions were used for band fitting to the original FTIR spectrum, with Gaussian-Lorentzian combinations, to de-

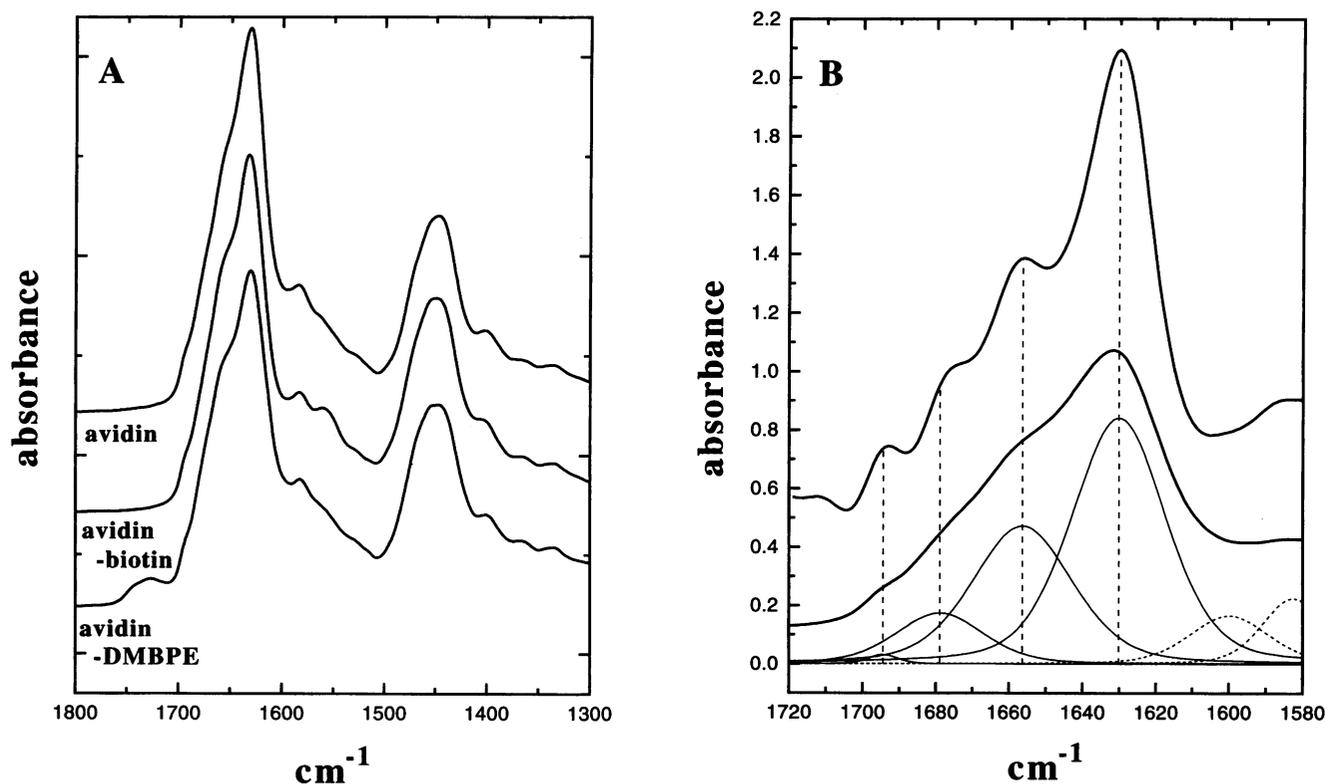


FIGURE 1 (A) FTIR spectra in the amide I, amide II, and lipid carbonyl region of avidin (*top*), avidin-biotin complex (*middle*), and avidin-DMBPE complex (*bottom*). (B) Expanded amide I region in the FTIR spectrum of avidin. (*Top*) Fourier-deconvoluted spectrum; (*middle*) original spectrum; (*bottom*) band fitting to the original spectrum, using Gaussian-Lorentzian combinations. Samples are in 10 mM Hepes/D₂O, pD 7.4, and spectra are recorded at 20°C.

termine the relative intensities of the component bands (see Fig. 1 B). Additional bands to low wave number (short-dashed curves) were also introduced to allow for overlap from outside the amide I region. The bands centered around 1630 and 1679 cm^{-1} correspond to (antiparallel) β -sheet and extended chain structures and make up approximately 66% of the total amide I intensity (cf. Byler and Susi, 1986). The band at 1656 cm^{-1} is in the range normally assigned to α -helical structures, but, because there is no evidence for the latter in the crystal structures of avidin (Pugliese et al., 1993; Livnah et al., 1993), it is likely to be contributed by other secondary structures. Recently the FTIR spectrum from a random coil peptide was found to occur at higher wave numbers than that usually attributed to unordered structures (Bauer et al., 1994). At least part of the band fitted at 1656 cm^{-1} may therefore correspond to disordered structures. Bands in this region have also been attributed to turns (Byler and Susi, 1986), which therefore could contribute additionally to the intensity of this band. The small band centered at 1694 cm^{-1} may also be due to β -turns (Krimm and Bandekar, 1986; Byler and Susi, 1986). Therefore, the major part of the secondary structure of avidin in solution is found to be antiparallel β -sheet and extended chain structures, with the remainder suggested to be contributed by unordered structure and turns.

Avidin-biotin complexes

As shown in Fig. 1 A, in the region 1520–1720 cm^{-1} the FTIR spectra of avidin and its complexes with biotin and with DMBPE are very similar. In Fig. 2, a comparison is made between the spectra of avidin and its complex with biotin (top panel). The difference spectrum obtained by subtracting the avidin spectrum from the spectrum of the avidin-biotin complex (middle panel) and the spectrum of biotin alone (bottom panel) are also shown in this figure. The spectra of avidin and the avidin-biotin complex are very similar, with small differences in the regions centered around 1554, 1630, and 1662 cm^{-1} . The differences at 1554 and 1662 cm^{-1} are due to the contribution of the complexed biotin molecule, as a comparison of the difference spectrum with the spectrum of biotin clearly shows. It is, however, important to note that the band corresponding to the ureido carbonyl is shifted to higher wave numbers for avidin bound to biotin (1662 cm^{-1}) relative to free biotin (1650 cm^{-1}). In contrast, the carbonyl band from the free carboxyl group occurs at approximately the same position (1554 cm^{-1}) for both free and bound biotin. The differences seen around 1630 cm^{-1} reflect some changes in the β -sheet and extended chain region of the avidin amide I absorption. From the shape of the difference spectrum, it can be seen that these correspond to a shift and most probably to a narrowing

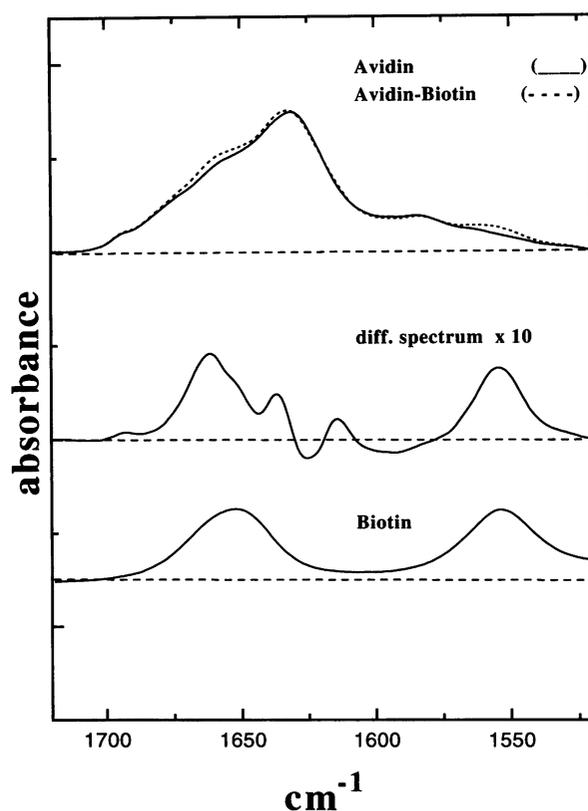


FIGURE 2 (Top) Undeconvoluted FTIR spectra in the amide I region of avidin (solid curve) and avidin-biotin complex (dashed curve). (Middle) Difference spectrum obtained by subtracting the avidin spectrum from the spectrum of the avidin-biotin complex (see Materials and Methods). (Bottom) FTIR spectrum of biotin. Samples are in 10 mM Hepes/D₂O, pH 7.4, and spectra are recorded at 20°C.

of the β -sheet band that results from changes in the peptide hydrogen bond stability rather than from a change in the population of β -sheet and extended chain structures. Although most of the intensity in the difference spectrum around 1662 cm^{-1} must come from the ureido carbonyl of biotin (cf. the carboxyl carbonyl intensity at 1550 cm^{-1}), it cannot be excluded that some change in the amide I band takes place also in this region.

Avidin-(biotin-lipid) complexes

FTIR spectra corresponding to those of Fig. 2 for the avidin-biotin complex are given in Fig. 3 A for the complex of avidin with the biotin-lipid DMBPE. The formation of the amide bond with the lipid headgroup amine in DMBPE shifts the carbonyl band of the biotin carboxyl to higher frequency ($\sim 1626\text{ cm}^{-1}$), resulting in a less well-resolved spectrum for DMBPE than for free biotin. This makes it more difficult to detect any shifts in the spectrum of the bound DMBPE, but there does appear to be a slight upward shift of the ureido carbonyl band in difference spectra constructed from the Fourier-deconvoluted spectra (data not shown).

Larger effects are seen in the region of the protein amide I band for the avidin-DMBPE difference spectrum in Fig. 3 A (bottom) than for that of the avidin-biotin complex (cf. Fig. 2). These additional spectral changes may be caused by the surface interaction of the basic avidin protein with the negatively charged DMBPE lipid assemblies. For this reason, FTIR spectra were recorded from a complex of avidin with the nonspecific negatively charged lipid DMPG, as a control. The corresponding FTIR spectra and difference spectrum are given in Fig. 3 B. For the complex with DMPG, the dominant feature of the difference spectrum occurs in the region of the major peak in the protein amide I band. This region of the spectrum is free of infrared bands from the DMPG lipid. Qualitatively, the shape of the difference spectrum resembles that of the avidin-DMBPE complex in this region. It thus appears that there are additional perturbations of the amide backbone H-bonds by surface interactions of avidin with the biotin-lipid that are not present in the complexes with free biotin. These result in a shift and possibly some redistribution of intensity in the region of the major amide I band characteristic of β -sheets and extended chain structures, but without appreciable change in the overall secondary structure of the protein (as evidenced by band fitting to the spectrum shown in Fig. 3 B, upper panel).

Temperature dependence of the FTIR spectra and heat denaturation of avidin

The temperature dependence of the deconvoluted FTIR spectra in the amide I region of avidin in the absence of biotin is given in Fig. 4. These data clearly show that the amide I band structure of avidin in solution remains nearly unaltered throughout the temperature range 20–74°C. Between 74 and 80°C, there is a large change in the structure of the amide I band, indicating a cooperative thermotropic transition in the structure of the protein. The ordered secondary structure of avidin, which is predominantly of the β -sheet type, collapses, and the spectra above this transition are characterized by two major bands centered at 1618 and 1683 cm^{-1} . Similar bands have been reported for other proteins, e.g., cytochrome *c* (Muga et al., 1991; Heimbürg and Marsh, 1993) and cholera toxin B subunit (Surewicz et al., 1990), on thermal denaturation. These bands have been attributed to extended intermolecular sheetlike structures or non-hydrogen-bonded edges of β -sheets (Muga et al., 1990, 1991; Heimbürg and Marsh, 1993). The cooperative nature of the denaturation transition is indicated in Fig. 5, in which the temperature dependence of the spectral changes is quantified from difference spectra constructed relative to the spectrum of the native protein recorded at 20°C. The sharp thermal transition centered at $\sim 77^\circ\text{C}$ is clearly seen.

Binding of biotin and of the biotinyl lipid DMBPE to avidin results in a dramatic increase in the thermal stability of the protein. For the complexes of avidin with either of these ligands there is no detectable change in the amide I

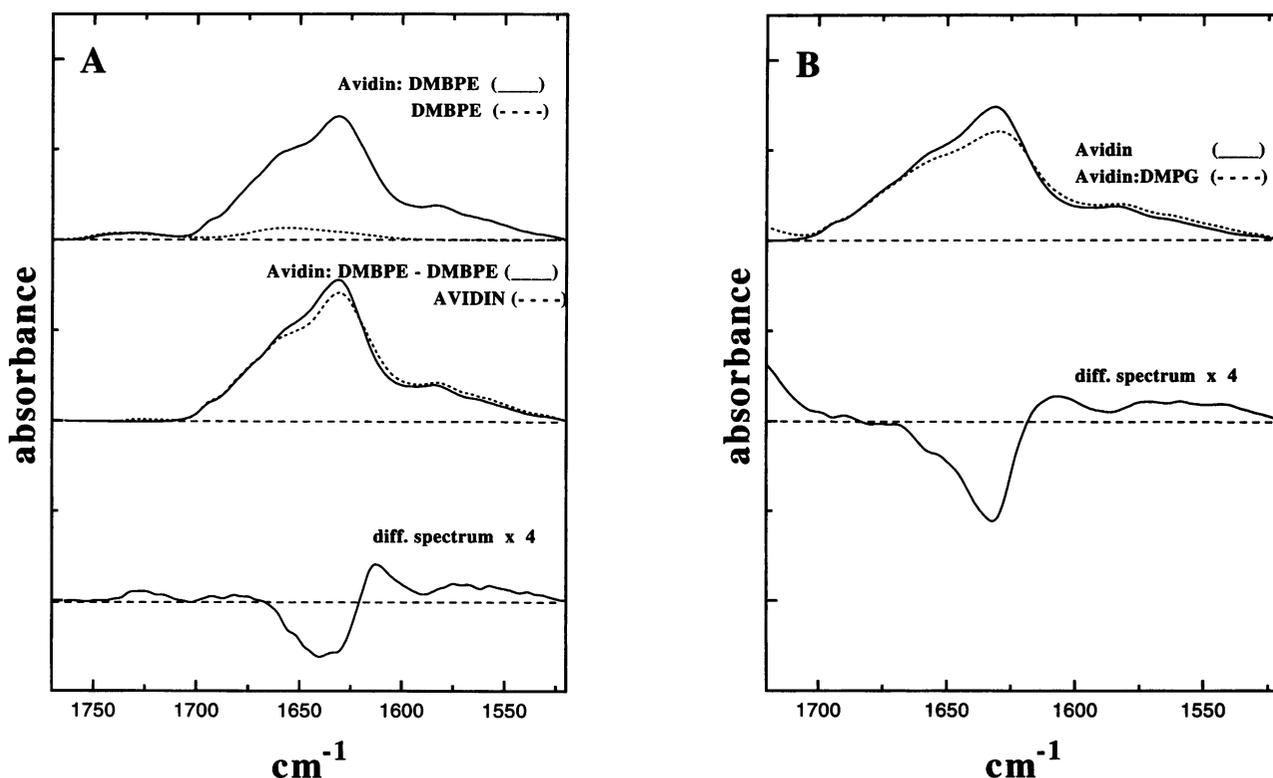


FIGURE 3 (A) (Top) Undeconvoluted FTIR spectra in the amide I and lipid carbonyl regions of the avidin-DMBPE complex (solid curve) and of DMBPE alone (dashed curve). Spectral heights are normalized in the lipid carbonyl region. (Middle) Difference spectrum obtained by subtracting the DMBPE spectrum from that of the avidin-DMBPE complex (solid curve) and FTIR spectrum of avidin alone (dashed curve), where both are normalized to the same integrated intensity. (Bottom) Double difference spectrum obtained by subtracting the two middle spectra to reveal the changes induced by binding to DMBPE. (B) (Upper curves) Undeconvoluted FTIR spectra in the amide I region of avidin (solid curve) and avidin-DMPG complex (dashed curve), where both are normalized to the same integrated intensity in the amide I region. (Lower curve) Difference spectrum obtained by subtracting the avidin spectrum from that of the avidin-DMPG complex. Samples are in 10 mM Hepes/D₂O, pD 7.4, and spectra are recorded at 20°C.

region of the FTIR spectra up to 93°C (data not shown). FTIR spectra could not be recorded above this temperature because of instrumental limitations.

DISCUSSION

Secondary structure of avidin

The secondary structure determination by FTIR spectroscopy yields ~66% β -sheet and extended chain structure for the native avidin in solution. This is significantly higher than the value of 55% determined by Honzatko and Williams (1982) using Raman spectroscopy. However, the value from FTIR is in reasonable agreement with the value of ~60% β -sheet and internally hydrogen-bonded extended chain structures determined from the crystal structure of avidin (Pugliese et al., 1993). Of this, 46–50% is involved directly in the eight-stranded antiparallel β -barrel of avidin and its deglycosylated form (Pugliese et al., 1993; Livnah et al., 1993). Core streptavidin, a related protein that shares 38% sequence identity with avidin (Aragarana et al., 1986) and also has a high affinity for biotin, has a very similar crystal structure (Weber et al., 1989).

Avidin-biotin and avidin-(biotin-lipid) complexes

Ligand binding is capable of inducing conformational changes in proteins and of affecting their stability. In a fluorescence study, Kurzban et al. (1989) have shown that the intrinsic fluorescence of avidin decreases by 34% and that binding biotin protected the tryptophan residues of avidin from quenching by external quenchers such as iodide and acrylamide. Honzatko and Williams (1982) have reported that there are not any significant changes in the secondary structure of avidin induced by biotin binding, as judged by Raman spectroscopy. A comparison of the FTIR difference spectrum from avidin with and without ligand with the spectrum of free biotin is given in Fig. 2. This reveals that, among the three regions where the avidin spectrum differs from that of the avidin-biotin spectrum, two regions, i.e., the bands around 1554 cm^{-1} and 1662 cm^{-1} , correspond to the carboxy carbonyl and primarily to the ureido carbonyl of the biotin, respectively, and therefore do not represent any large changes in the secondary structure of the protein. The differences seen in the wave-number range 1610–1635 cm^{-1} are in the amide I region corresponding to β -sheet and extended chain structures and

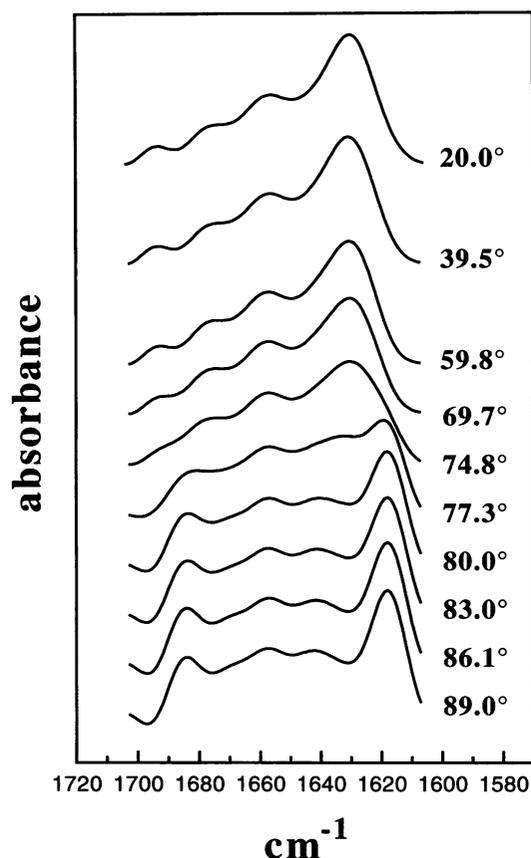


FIGURE 4 Temperature dependence of the Fourier self-deconvoluted FTIR spectra of avidin in 10 mM Hepes/D₂O, pD 7.4. The temperature (°C) at which each spectrum was recorded is shown at the right.

therefore reflect some changes in this part of the protein. The form of the difference spectrum in this region indicates that changes correspond to a shift, and possibly a narrowing, of the β -sheet band rather than to a change in its overall intensity. This suggests that there are no large changes in the secondary structure content of avidin on binding biotin. The shift of the band does, however, indicate an alteration in stability of the hydrogen bonds within the β -sheet and extended chain structures.

The complex of avidin with assemblies of the biotin-lipid DMBPE shows somewhat larger changes in the amide I region of the FTIR spectrum than does the complex with free biotin (see Figs. 2 and 3 A). Again these changes correspond to perturbation of the β -sheet and extended chain structures rather than to a change in the overall secondary structural content. Comparison with the amide I region in the FTIR spectrum of avidin bound electrostatically to the nonspecific anionic lipid DMPG (Fig. 3 B) indicates that this additional perturbation arises from surface interactions with the lipid aggregates.

A comparison of the carbonyl bands of biotin in the free state and in the bound form obtained from the difference spectrum shown in Fig. 2 shows that, whereas the stretching frequency of the carboxy carbonyl is the same for both free

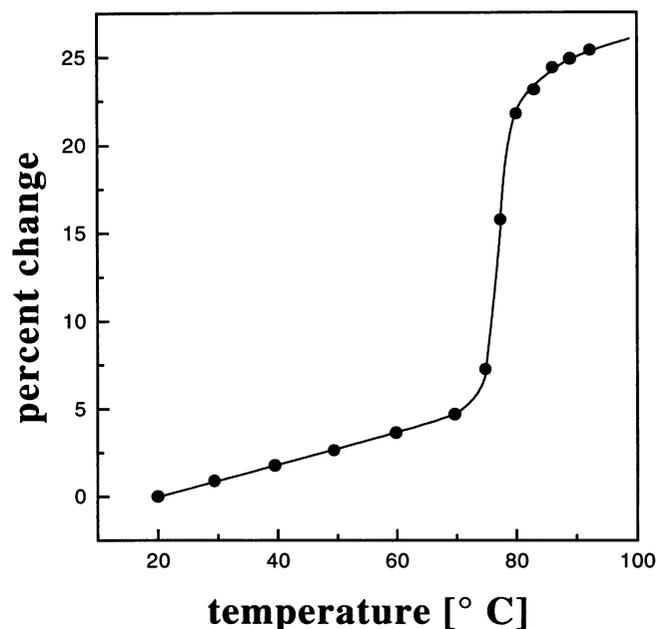


FIGURE 5 Temperature dependence of the total area above the baseline in the FTIR difference spectra of the amide I band of avidin in 10 mM Hepes/D₂O, pD 7.4. Difference spectra were obtained by subtracting a deconvoluted reference spectrum recorded at 20°C from the deconvoluted spectra recorded at various temperatures with areas normalized to 100 \times cm^{-1} .

and bound forms, that of the ureido carbonyl is shifted to higher wave numbers. This suggests that the C—O bond of the ureido carbonyl is shorter in length in the bound state than in the free state, as a result of interaction with the protein. It is known that the lengths of the C—O bond of the urea carbonyl can vary in the presence of polarizing groups (Blessing, 1983). The biotin molecule can be represented as canonical forms 1–3 (see Fig. 6), where the nitrogens bear a positive charge and the oxygen bears the negative charge. In addition, the keto-enol-like tautomeric forms 1, 4, and 5 will also contribute to the polarizability of the ureido carbonyl in the free biotin. From crystallographic studies of the avidin-biotin complex as well as of the related streptavidin-biotin complex it was found that the amide protons of the biotin ureido group are hydrogen bonded to the protein amino acid sidechains (Pugliese et al., 1993; Livnah et al., 1993; Weber et al., 1992). Hence, the contribution of the tautomeric forms 4 and 5 to the structure of the ureido carbonyl will be smaller in the bound state, resulting in a small decrease in the bond length that is reflected in the infrared spectrum as a shift of the ureido carbonyl band to higher wave numbers.

The crystallographic studies of the avidin-biotin complex (Livnah et al., 1993; Pugliese et al., 1993) and the streptavidin-biotin complex (Weber et al., 1992) have shown that the carbonyl oxygen of the ureido group of biotin is hydrogen bonded to three different hydrogen atoms of the protein. The arrangement of the three hydrogen atoms and the carbonyl carbon around the oxygen has a tetrahedral

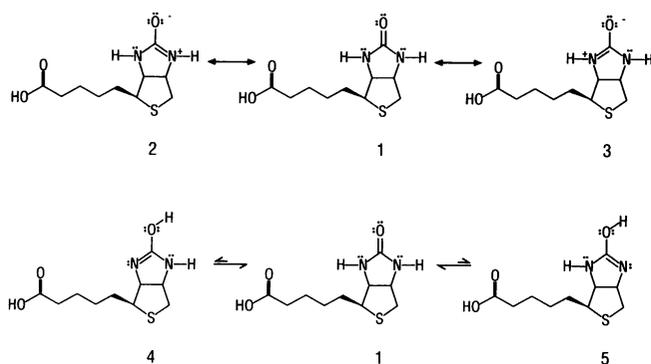


FIGURE 6 Canonical forms of the biotin molecule (1–5); the nitrogens bear a positive charge and the oxygen bears the negative charge. The keto-enol-like forms (1, 4, and 5) also contribute to the polarizability of the ureido carbonyl in the free biotin.

symmetry. This was interpreted as indicative of sp^3 character in the ureido C—O bond, the polarization of which results in formation of an oxyanion. In contrast to this, the results obtained here suggest that the sp^3 character is likely to be less in the avidin–biotin complex than in biotin free in solution.

Thermal denaturation of avidin

Avidin is a particularly stable protein whose stability is increased further by ligand binding, as evidenced by the elevation of the denaturation temperature on complexing with biotin (Donovan and Ross, 1973). We investigated the structural nature of the thermal denaturation process for native avidin as well as with its complexes with biotin and with the biotin lipid DMBPE. It is known that interaction with membranes can lead to changes in protein stability (see, e.g., Heimburg and Marsh, 1993); therefore the specific changes that might occur on binding avidin to biotin-PE lipids are of particular interest. The data shown in Fig. 5 reveal that the structural changes that occur on thermal denaturation of avidin take place cooperatively at $\sim 77^\circ\text{C}$. This value is in reasonably close agreement with the calorimetric denaturation temperature of 85°C determined at moderately fast heating rates (Donovan and Ross, 1973). The changes taking place in the FTIR spectra of avidin on denaturation indicate the loss of the native β -sheet structure (Fig. 4). Concomitantly, infrared bands appear in a region that has been attributed to the formation of intermolecular hydrogen bonding (Muga et al., 1990; Surewicz et al., 1990), and a band in the region characteristic of unordered structures becomes more pronounced. The formation of intermolecular aggregates is consistent with the increase in turbidity of avidin solutions that is found in the region of the denaturation temperature (Donovan and Ross, 1973).

The avidin–biotin and avidin–DMBPE lipid complexes did not show any changes in their FTIR spectra up to 93°C , our experimentally accessible range of temperature. Nor was a denaturation transition detected calorimetrically for avidin bound to the DMBPE biotin–lipid up to 110°C (data

not shown). These results indicate that the large stabilization induced in avidin on binding the biotin moiety is present also on binding to assemblies of the DMBPE lipid. Any destabilization by membrane binding per se, such as is seen with cytochrome *c* (Muga et al., 1991; Heimburg and Marsh, 1993), is outweighed by the specific effects of the biotin ligand. The origin of the stabilization most probably lies in the increased stability of the hydrogen bonds in the β -sheet structures, a conclusion that is supported by the infrared difference spectra (see above).

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