^{13}C NMR studies of the binding of medium-chain fatty acids to human serum albumin

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Abstract Binding of the medium-chain fatty acids (MCFA), octanoic (OCT) and decanoic (DEC) acid, to human serum albumin (HSA) has been studied by ^{13}C NMR spectroscopy. NMR spectra at 35°C showed an apparently homogeneous binding environment (a single, narrow resonance for the ^{13}C-enriched carboxyl carbon) at different mole ratios and pH values. Changes in the chemical shift of this peak with mole ratio and protein concentration demonstrated rapid equilibration ($\leq$ msec) of bound and unbound MCFA and permitted a direct quantitation of bound/unbound MCFA. Spectra of OCT/HSA mixtures at 6°C revealed at least three distinct binding sites that fill sequentially. The observed heterogeneity of binding at low temperature, compared to 35°C, is attributed to a slower exchange rate of OCT between binding sites. The highest affinity sites for both OCT and DEC have properties similar to those of binding sites for longer-chain fatty acids, such as the close proximity of the fatty acid carboxylate to basic amino acid residue(s). Interestingly, chemical shift data showed that the first mole of OCT and DEC either bind differently to the same site or bind to different sites on HSA. The rapid desorption of MCFA from HSA binding sites has implications for dietary regimens with medium chain triglycerols.—**Kenyon, M. A., and J. A. Hamilton.** ^{13}C NMR studies of the binding of medium-chain fatty acids to human serum albumin. *J. Lipid Res.* 1994. **35:** 458–467.

Supplementary key words octanoic acid • decanoic acid • binding affinities • exchange rates • parenteral feeding

Human serum albumin (HSA) is a key transport protein in plasma that plays an important role in lipid metabolism by virtue of its high-affinity and high-capacity binding of free (unesterified) fatty acids (FA). The physiological importance of plasma free FA and the pathological effects resulting from abnormally high levels of free FA (1) have encouraged ongoing investigations of the binding and transport of free FA by albumin. The high-resolution X-ray structure of FA-free HSA (2) shows elegantly and in great detail the loops and domains that were predicted by numerous investigators (3). Nevertheless, molecular details of the binding of FA, particularly those of medium-chain length (8 or 10 carbons), are incomplete. Although medium-chain fatty acids (MCFA) normally constitute a very minor fraction of the FA in plasma, interest in the binding of MCFA exists as the levels of these FA can be greatly elevated in certain disease states (4), in patients fed intravenous medium chain triacylglycerols (4–6) and in infants treated for low birth weight (7). Interest in the binding of MCFA to HSA also stems from the observation that these acids may compete with certain drugs and with tryptophan for binding sites on HSA (8–11).

Interactions of MCFA with albumin have previously been probed by analysis of equilibrium binding data in the presence and absence of competing ligands (9, 12) and as a function of pH (12). However, equilibrium binding studies do not differentiate multiple binding sites with similar affinities and do not provide direct information about structural features of the binding sites. Additional strategies have attempted to locate MCFA binding sites on serum albumin by covalent modification of HSA (13) and by fragments of bovine serum albumin (BSA) (14, 15).

A more recent approach for examining interactions of FA with albumin has been ^{13}C NMR spectroscopy. This spectroscopic approach, which uses native FA with a nonperturbing modification (^{13}C enrichment), can provide information about molecular interactions in individual binding sites, in contrast to methods that report on average behavior. New information has been provided about ionic interactions between the FA carboxyl group and the amino acids in the binding sites on BSA, about the location of high affinity sites on BSA for long-chain fatty acids, and about the dependence of interactions on FA chain length (16–20). Like classical approaches, ^{13}C NMR spectroscopy has provided less information about the binding of MCFA to albumin (21) than about long-chain FA and, to date, most studies have focused on BSA rather than HSA. In this study, interactions of MCFA [OCT (8 carbon) and DEC (10 car-

Abbreviations: MCFA, medium-chain fatty acids; HSA, human serum albumin; FA, fatty acid; OCT, octanoic acid; DEC, decanoic acid; BSA, bovine serum albumin; CD, circular dichroism; UV, ultraviolet; TMS, tetramethylsilane.

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bon)] with HSA were investigated by high-resolution ^{13}C NMR spectroscopy. NMR measurements (primarily chemical shift and lineshape) were made as a function of pH, temperature, and the mole ratio of FA to albumin. Our results illuminate molecular aspects of MCFA binding to HSA.

MATERIALS AND METHODS

Materials

Lyophilized, crystallized HSA (A3782, primarily Lot 127F-9310) was obtained from Sigma Chemical Co., St. Louis, MO. The essentially FA-free HSA contained less than 0.01 mole of FA per mole of protein as determined by gas–liquid chromatography. Ninety percent ^{13}C carboxyl-enriched OCT and DEC used in this study were purchased from CIL, Cambridge, MA.

Sample preparation

A measured amount of HSA was dissolved in 0.56% KCl and the protein concentration was determined from the absorbance at 279 nm (22) of filtered 1:100 dilutions in 7.5 mM KCl; the extinction coefficient used was 0.55 ml $mg^{-1}cm^{-1}$ (23). The protein concentration was ~93 mg/ml for all NMR samples unless otherwise stated. OCT/HSA and DEC/HSA complexes were prepared with aqueous potassium OCT and with aqueous potassium DEC, respectively. The concentration of the FA, dissolved in chloroform–methanol 2:1, was determined by measuring dry weights on an electrobalance (Cahn model 25, Cerritos, CA). The aqueous solutions of potassium FA were made by combining a known amount of ^{13}C-enriched FA with 1.2 eq of base (1 N KOH, 0.1 N KOH). Sodium could be substituted for potassium, yielding identical results. FA/HSA samples were made by the addition of an appropriate amount of potassium FA (depending on the desired mole ratio of FA to HSA) directly to a measured volume of aqueous protein (1.4–1.6 ml). To increase the mole ratio, FA was added directly to the FA/HSA complex. OCT/HSA and DEC/HSA mixtures were gently vortexed and these samples were adjusted initially to a pH of 7.4. During the course of some experiments, the pH of the samples was changed by adding small amounts of KOH (0.1 N, 1 N) or HCl (0.1 N, 1 N) directly to the NMR tube, using a microliter syringe. The pH values were measured by a Beckman model 3560 pH meter equipped with a 5-mm diameter microelectrode. pH values for the FA/HSA samples before and after NMR analysis differed by ≤ 0.2 pH units. Final pH values are reported.

^{13}C NMR spectroscopy

A Bruker WP-200 spectrometer operating at 4.7 T (50.3 MHz) was used, unless otherwise noted, to obtain ^{13}C NMR spectra (16). Selected spectra were obtained with a Bruker AMX 300 spectrometer operating at 7.05 T (75 MHz for ^{13}C). Spectra were obtained with 16K time domain points and a 10 KHz (4.7 T) or 15 KHz (7.05 T) spectral width. Aqueous FA-albumin complexes included 100 μl of D_2O for a lock and a shim signal. The NMR sample tubes supported an insert containing tetramethylsilane (TMS) in $CDCl_3$. All chemical shifts were measured with respect to this external reference. The uncertainty of all chemical shift values reported is ± 0.05 ppm, based on multiple measurements of samples with similar compositions prepared and analyzed at different times. Each NMR experiment reported in this study was repeated one to three times. Data shown are representative data. The temperature dependence of the reference signal was estimated to be < 0.1 ppm in the temperature range investigated. Temperature was controlled (± 1–$2°C$) with a Bruker B-VT-1000 variable temperature unit. The internal temperature of selected samples was measured as follows. After the sample had equilibrated in the magnet for ~10 min at a fixed temperature that was regulated by the variable control unit, the sample was removed (time zero). A thermocouple was immediately inserted in the sample and several temperature values were recorded at 15-sec intervals. The temperature at time zero (corresponding to the true sample temperature) was obtained by extrapolation of the linear relationship of temperature versus time. A temperature calibration curve was constructed from measurements at several temperatures.

Spin lattice relaxation time was measured for selected samples by the fast inversion-recovery method (24). Pulse intervals for obtaining standard spectra were generally chosen for optimal signal to noise ratios ($\sim 1 \times T_1$) rather than for equilibrium intensities ($5 \times T_1$). Deconvolution of spectra was performed with NMR1 (New Methods Research, Inc., East Syracuse, NY).

CD spectroscopy

Near-UV CD spectra were recorded using a Cary 61 CD spectropolarimeter (Varian, Palo Alto, CA). Three continuous spectra per sample were recorded in the wavelength range of 320–260 nm. Data points were analyzed at 1-nm intervals; trough depth measurements were made with respect to the baseline value. Molar ellipticity values $[\theta]$, in units of deg · cm^2/dmol, were calculated by the standard equation for a path length of 0.02 cm (25).

For temperature-dependent CD spectra, the sample temperature was maintained by circulating ethylene glycol–water through the lamp compartment by means of a thermostated refrigerator-heater bath (NESLAB, Portsmouth, NH). Temperature was measured to within 0.1°C by means of a copper-constantan thermocouple positioned in contact with the CD cell. With each temperature change CD spectra were recorded after a short time interval (~15 min) to allow the sample to equilibrate at the desired temperature setting.

RESULTS

NMR studies at 35°C

^{13}C NMR spectra for mixtures of ^{13}C carboxyl-enriched OCT or DEC with HSA were obtained as a function of mole ratio of FA to protein at T = 34.5°C and pH = 7.40 ± 0.15. The MCFA carboxyl carbon gave a single peak at all mole ratios; the intensity of this peak increased relative to that of protein peaks (e.g., the broad carbonyl at ~170–180 ppm) with increasing FA to HSA mole ratio. Under similar experimental conditions, FA with a chain length of ≥12 carbons in the presence of HSA or BSA give rise to multiple narrow signals whose individual intensities increase with increasing mole ratio (18, 26). For OCT and DEC the chemical shift of the single carboxyl peak showed a dependence on mole ratio (**Fig. 1A**). For OCT/HSA mixtures, the chemical shift increased steadily with increasing mole ratio of FA/HSA, from 181.80 ppm (1:1 OCT/HSA) to 182.83 ppm (15:1 OCT/HSA). The value of the chemical shift approached but did not reach that of unbound OCT (184.32 ppm; see below). In contrast, the chemical shift of DEC/HSA mixtures showed only a small dependence on mole ratio except at very high ratios (Fig. 1A). Between a 3:1 and 11:1 mole ratio of DEC/HSA, there was a small (0.28 ppm) linear increase in chemical shift. Above 11:1 DEC/HSA, a progressive shift to higher ppm was seen.

The dependence of the FA carboxyl chemical shift on protein concentration was examined at 34.5°C and pH = 7.4 by diluting samples with a fixed mole ratio of OCT or DEC to albumin (3:1 and 10:1) from 90–100 mg protein/ml to the lowest concentration feasible for NMR studies (10–20 mg/ml). In all cases a single, narrow resonance from the FA carboxyl carbon was observed. The FA chemical shift for the OCT/HSA system was more dependent on protein concentration than that of the DEC/HSA system (Fig. 1B). The OCT carboxyl peak shifted downfield (to higher ppm) with decreasing HSA concentration, from 182.05 ppm (92 mg/ml) to 182.50 ppm (23 mg/ml) for a 3:1 OCT/HSA complex and from 182.30 ppm (98 mg/ml) to 183.15 ppm (12 mg/ml) for a 10:1 OCT/HSA complex. The chemical shift for the 3:1 DEC/HSA complex remained constant at 182.20 ppm for all HSA concentrations, whereas the 10:1 DEC/HSA mixture showed a slight increase in chemical shift (from 182.30 ppm to 182.45 ppm) at the lowest concentration (12 mg/ml).

NMR spectra obtained as a function of pH can provide important information about binding interactions from the ionization behavior of FA in the presence of protein (16, 19). Therefore, ^{13}C NMR spectra were obtained at 34.5°C as a function of pH for several mole ratios of OCT/albumin (1.5:1, 3:1, and 5:1). These spectra showed a single resonance under all conditions. **Fig. 2** compares the titration behavior of OCT in the presence of HSA (3:1

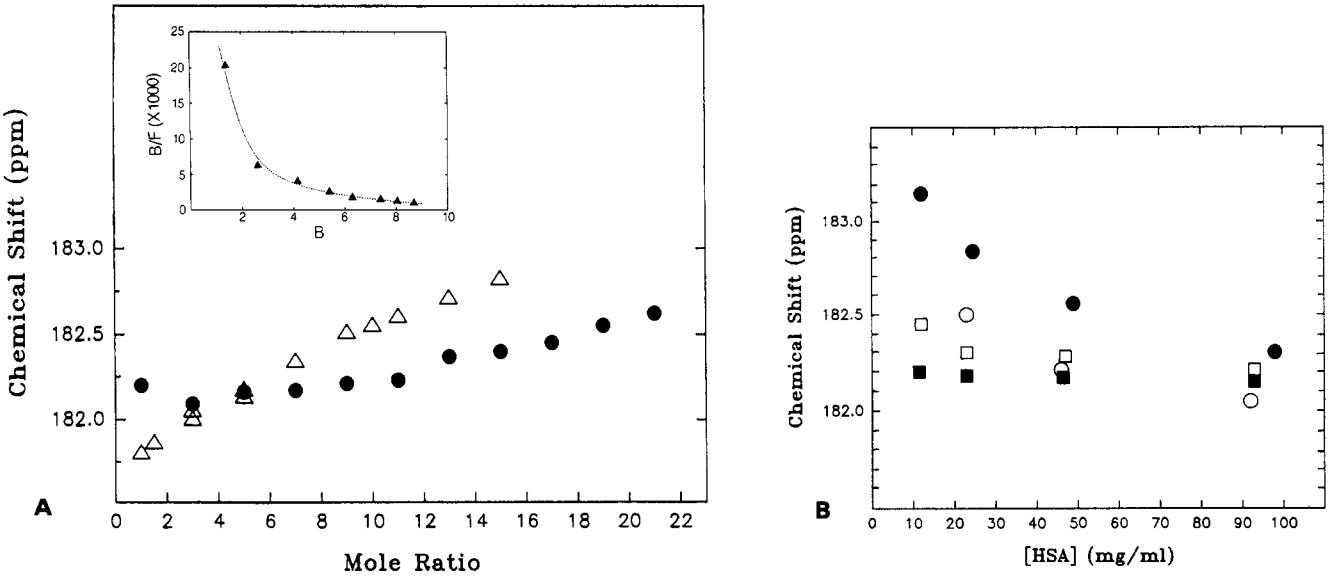

Fig. 1. A: Plot of FA carboxyl ^{13}C chemical shift versus mole ratio at T = 34.5°C and pH = 7.4 ± 0.2. The mole ratio represents the stoichiometric amount of FA and albumin in the mixture. Filled circles represent DEC/HSA mixtures; unfilled triangles correspond to OCT/HSA mixtures. All samples had a protein concentration of 93 mg/ml. The inset shows a Scatchard plot of the mole ratio data for a three-term fit. B: Plot of FA carboxyl ^{13}C chemical shift versus HSA concentration at T = 34.5°C and pH = 7.4 ± 0.2. Open and closed circles represent 3:1 and 10:1 OCT/HSA mixtures, respectively. Closed and open squares correspond to 3:1 and 10:1 DEC/HSA mixtures, respectively. Conditions for obtaining NMR spectra were as follows: spectral accumulations ranged from 250 to 1000 for DEC/HSA and 6800 to 10,000 for OCT/HSA over 16,384 time domain points with a pulse interval of 2.0 sec. Chemical shift values were measured with respect to an external reference (tetramethylsilane) in units of ppm (parts per million).

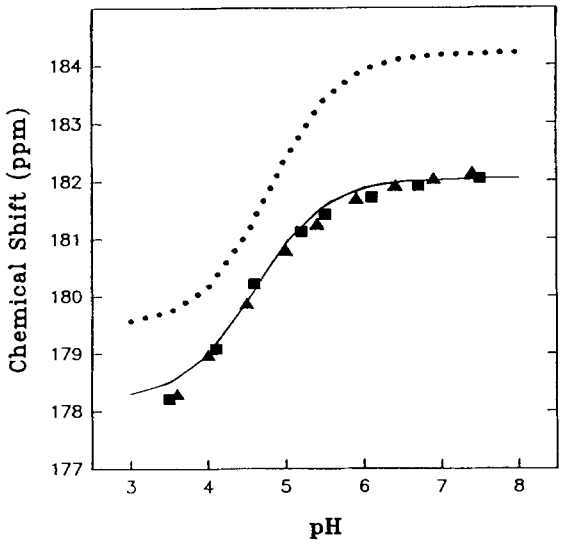

Fig. 2. Plot of OCT carboxyl ^{13}C chemical shift versus pH at T = 34.5°C and mole ratios of 3:1 (filled square) and 5:1 (filled triangle) OCT/HSA. The protein concentration of all samples was 92-93 mg/ml. The solid line is the theoretically calculated Henderson-Hasselbach curve for a 3:1 OCT/HSA mixture. The dashed curve is the ^{13}C NMR titration of aqueous octanoic acid at 1.6 mM and 40°C (20).

and 5:1 mole ratios) with that of OCT in the absence of protein. The plot of chemical shift versus pH closely follows the sigmoidal Henderson-Hasselbach behavior that is characteristic of an acid–base titration (27). The apparent pKa value for OCT/HSA complexes was the same as the pKa of aqueous OCT (pKa = 4.6). However, the titration curve for OCT with HSA is shifted to lower ppm compared to the curve for unbound OCT, a shift that reflects partitioning of MCFA into a less hydrophilic environment than water (i.e., interactions of OCT with the protein). pH-dependent spectra (not shown) were also obtained at 34.5°C for DEC/HSA complexes at 1.5:1 and 3:1 mole ratios. For the 1.5:1 mole ratio, the chemical shift was monitored between pH 8.1 (182.16 ppm) and pH 6.1 (182.01 ppm), below which the carboxyl signal became too broad to measure the chemical shift. For the 3:1 mole ratio, it was possible to measure the chemical shift at lower pH values. The chemical shift decreased from 182.26 ppm at pH 8.4 to 181.21 ppm at pH 4.7. These data for the 3:1 DEC/HSA complex suggest a titration of DEC similar to that for OCT in the presence of HSA (Fig. 2).

NMR studies at low temperature

In the above NMR studies at 35°C, the FA carboxyl group appeared to experience a single "binding" environment under various experimental conditions: different FA/HSA mole ratios, pH values, and protein concentrations. These results could mean that binding sites for MCFA are not structurally heterogeneous (unlike the case for long-chain FA) or that the NMR experiments did not detect heterogeneous sites. The latter case could occur if FA in different binding sites exchange rapidly to produce an apparent "single" site, which has been shown to occur with binding of MCFA to BSA (21). As it was possible to detect multiple binding sites for MCFA on BSA at lower temperatures (21), a similar strategy was applied to HSA complexes with OCT and with DEC at fixed mole ratios of MCFA/HSA (1.5:1, 3:1, 5:1) and at pH 7.5 ± 0.1. **Fig. 3** illustrates ^{13}C NMR spectra (FA carboxyl region) for a 3:1 mole ratio complex of OCT/HSA at five temperatures. At the higher temperatures (41.5°C and 34.5°C), a single narrow peak (181.97 ppm and 182.01 ppm, respectively) was observed from the ^{13}C-enriched MCFA. As the temperature was decreased, the carboxyl resonance first broadened (Fig. 3C) and then separated into two major signals (Fig. 3D). At 6.5°C (Fig. 3E) the spectrum exhibited one signal at higher ppm and the other at lower ppm relative to the single signal at 35°C, characteristic of exchange (see below). A spectrum at ~6° obtained at 75 MHz (not shown) did not show improved resolution relative to the spectrum at 50 MHz (Fig. 3E).

Temperature-dependent spectra for a 3:1 DEC/HSA

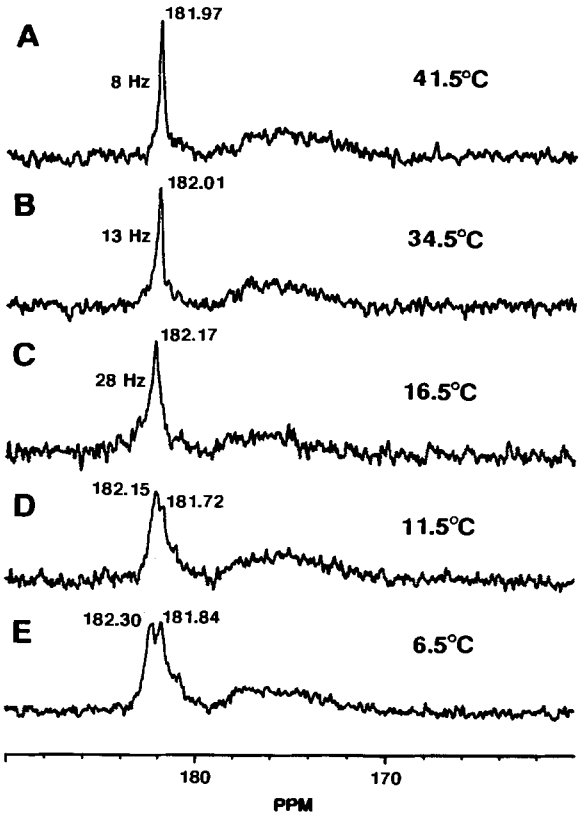

Fig. 3. The carboxyl and carbonyl regions of ^{13}C NMR spectra for a 3:1 mole ratio of 90% [1-^{13}C]OCT/HSA complex at various temperature values: A) T = 41.5°C after 1000 accumulations; B) T = 34.5°C after 1000 accumulations; C) T = 16.5°C after 4000 accumulations; D) T = 11.5°C after 8000 accumulations; and E) T = 6.5°C after 1186 accumulations. All spectra were recorded with a pulse interval of 2.0 sec and processed with a line broadening 4.0 Hz. The sample pH was 7.4 ± 0.1 and the protein concentration was 93 mg/ml.

are shown in **Fig. 4A.** For this mole ratio, as well as the two other mole ratios investigated (1.5 and 5.0), a single carboxyl resonance was observed at T > 21°C (Fig. 4A, top spectrum). As the temperature was decreased (e.g., 20.5°C), the chemical shift of the predominant peak shifted downfield slightly and a second small signal was seen upfield from this signal (Fig. 4A, middle spectrum). At 6.5°C the major signal was at 182.30 ppm and a broader, less defined signal was seen between 181.64 ppm and 181.15 ppm (Fig. 4A, bottom spectrum). A low temperature spectrum at higher field (75 MHz) showed evidence of a third signal at 181.96 ppm between the two separated peaks (Fig. 4A, right side). The carboxyl spectral region for 1 mole DEC/mole HSA at T = 6.5°C is shown in Fig. 4B (top spectrum). The spectrum of FA-free HSA (Fig. 4B, bottom spectrum) contains signals around 181.09 ppm from amino acid carboxyl groups that are shifted away from the intense, broad envelope of carbonyl/carboxyl resonances, similar to the case for BSA (16). Subtraction of the protein component from the spectrum for 1:1 DEC/HSA revealed a narrow peak centered at 182.28 ppm (Fig. 4B).

Temperature-dependent spectra in the same temperature range as for OCT and for DEC were obtained for the 12-carbon fatty acids (dodecanoic or lauric). These spectra gave three resonances (181.8, 182.0, and 182.3) for a 3:1 mole ratio of FA/HSA at high temperature, as shown previously (26). There was little effect of temperature in the carboxyl spectrum. Therefore, the temperature-dependent changes described above are specific for MCFA and are not due to protein aggregation or other general temperature-dependent structural changes.

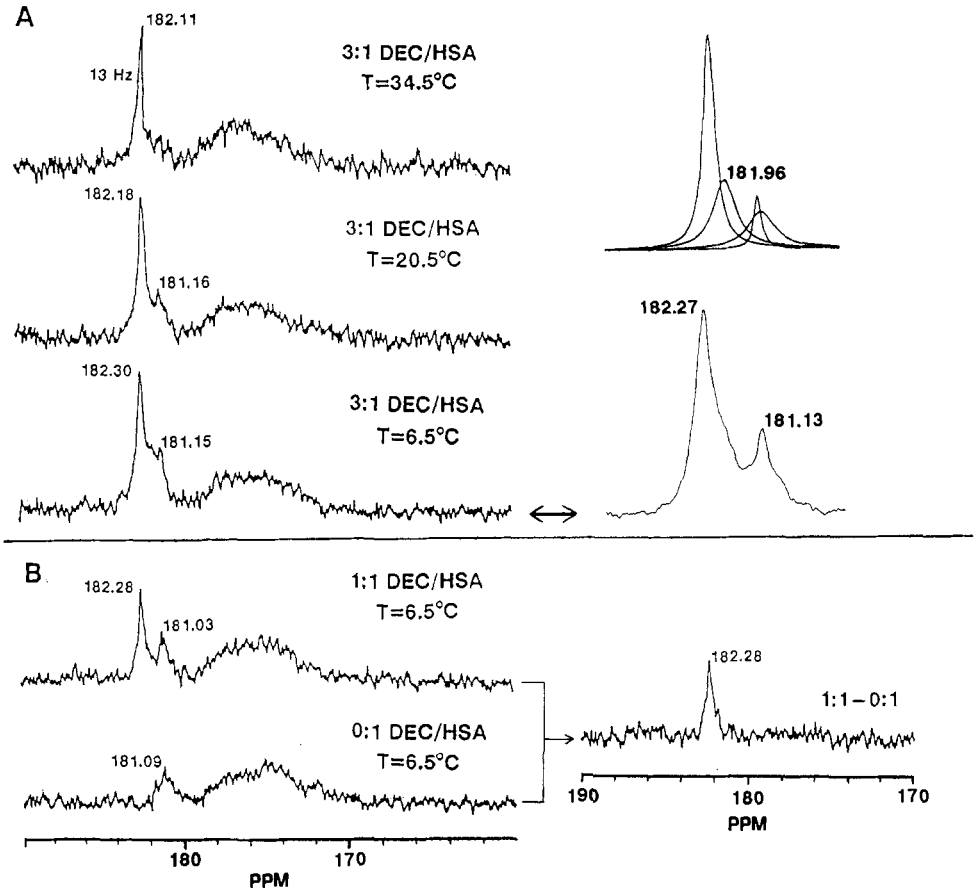

Fig. 4. The carboxyl and carbonyl regions of ^{13}C NMR spectra for A) a 3:1 mole ratio of 90% [1-^{13}C]DEC/HSA mixture at various temperatures: T = 34.5°C, 1200 scans (top spectrum); T = 20.5°C, 2000 scans (middle spectrum); and T = 6.5°C, 3003 scans (bottom spectrum) and for B) a 1:1 mole ratio of 90% [1-^{13}C]DEC/HSA sample at 6.5°C, recorded after 4000 scans (top spectrum); a spectrum of FA-free HSA at 6.5°C (bottom spectrum); and the difference of the 1:1 and 0:1 mole ratio of DEC/HSA (right). A spectrum (4000 scans) of a 3:1 DEC/HSA sample at 6.5°C recorded at a higher field (7.05 T) and the corresponding deconvolution spectrum are shown in panel A (right column). The deconvolution shows, in addition to the narrow signals at 182.27 ppm and 181.13 ppm, a broader signal at 181.96 ppm, representing a third binding environment for DEC, and a weaker broad signal from protein carboxylates underlying the FA carboxyl signal at 181.13 ppm (see panel B, bottom spectrum). All other NMR and sample conditions were the same as those stated in Figs. 1 and 3.

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