Stereospecific ^{1}H and ^{13}C NMR Assignments of Crotonyl CoA and Hexadienoyl CoA: Conformational Analysis and Comparison with Protein−CoA Complexes

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Abstract: Stereospecific ^{1}H and ^{13}C resonance assignments of hexadienoyl-CoA and crotonyl-CoA have been determined. The two diastereotopic methyl groups at the C2″ carbon were assigned via transferred nuclear Overhauser effect experiments on the complex of hexadienoyl-CoA with the enzyme enoyl-CoA hydratase. The two diastereotopic 1″ protons were assigned using heteronuclear multiple-bond correlation experiments in conjunction with rotating frame nuclear Overhauser spectroscopy. This represents the first set of complete stereospecific assignments for any CoA derivative. The assignments allow a detailed quantitative conformational analysis of the uncomplexed form of the molecule. In particular, the relative population of the various rotamers about the C1″−C2″ and the C2″−C3″ bonds have been determined. The database of protein-bound CoA structures has been surveyed and used to compare the structure(s) of CoA in protein−CoA complexes with the conformational preferences of free CoA.

Introduction

Coenzyme A (CoA) and its derivatives play a central role in metabolism and approximately 4% of all known enzymes utilize CoA derivatives as substrates. Despite its importance, there have been surprisingly few detailed NMR studies of CoA or CoA derivatives. Early work used classical one-dimensional (1D) methods to assign the proton[1−4] and carbon[5,6] NMR spectra. More recently, Anderson and co-workers used two-dimensional (2D) experiments in conjunction with 1D ^{1}H NOE difference experiments to assign the proton and carbon spectra of CoA.[7] In their study, tentative stereospecific assignments of the two C2″ (Figure 1A) diastereotopic methyl groups of CoA were proposed based upon 1D ^{1}H nuclear Overhauser effect (NOE) difference data and analysis of crystal structures of CoA−protein complexes.[7] Unfortunately, it was not possible to stereospecifically assign the diastereotopic 1″ protons using these methods, and the assignment of these protons has not yet been reported in the literature. In the absence of stereospecific assignments, it is not possible to uniquely define the populations of the three noneclipsed rotamers about the C1″−C2″ or about the C2″−C3″ bonds (Figure 1B). Early conformational analysis of this portion of the molecule was also hindered by the fact that there are no three-bond proton−proton coupling constants that can be used to help define the conformation about these bonds.

The portion of CoA including the C1″−C2″ and C2″−C3″ bonds is a particularly interesting and important region of the molecule. It contains the only chiral center of the pantoic acid portion of CoA located at the 3″ position and also includes the 2″ geminal dimethyls. The methyl substituents at the 2″ carbon are expected to play an important role in controlling the dynamics and conformational tendencies of CoA. It has been suggested that the geminal dimethyls may have played an important role in the prebiotic synthesis of CoA.[8] Jencks and co-workers have demonstrated that the nucleotide and pantoic acid portions of CoA have separate roles in catalysis.[9,10] Their detailed studies of the interaction of CoA and truncation analogues of CoA with the enzyme 3-oxoacid-CoA transferase have demonstrated that much of the binding energy that stabilizes the covalent intermediate is due to interactions between the nucleotide domain of CoA and the enzyme. In contrast, binding to the pantoic acid region destabilizes the covalent enzyme intermediate but favorable interactions involving the pantoic acid domain occur in the transition state for the second half-reaction. The removal of the destabilizing interactions combined with the generation of favorable interactions in the transition state results in a large reduction in the activation barrier for the second half-reaction. Jencks and co-workers were able to demonstrate that these important binding interactions between CoA and the enzyme are localized to the 2″,2″-dimethyl-3″-hydroxyl portion of CoA.

In this work, we assign the two diastereotopic C1″ protons and the two diastereotopic C2″ methyls. With these stereospe-

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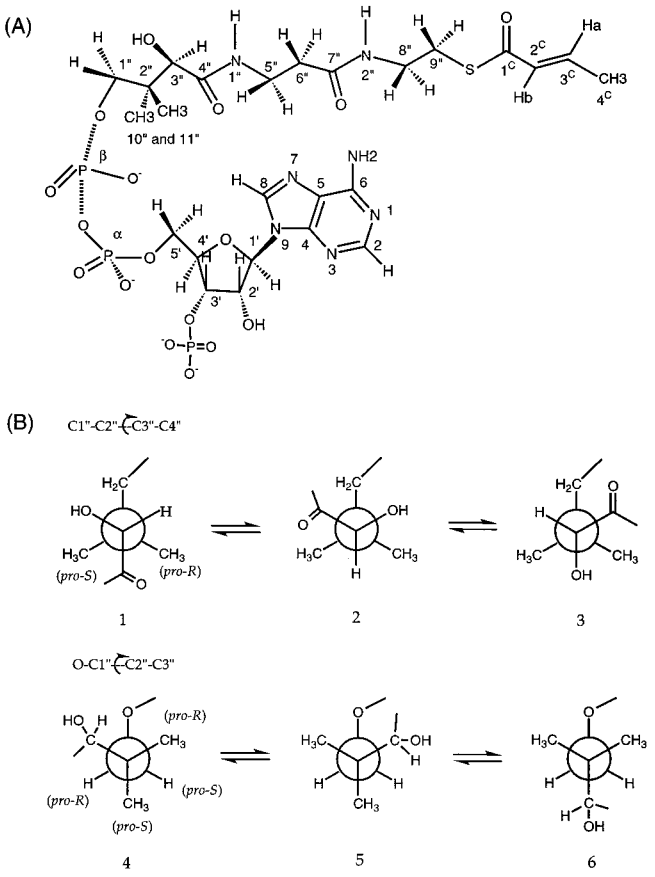

Figure 1. (A) Schematic drawing of the covalent structure of crotonyl-coenzyme A. The numbering system used in this paper is shown. (B) Newman projections of the three low-energy rotamers about the C2″−C3″ and the C1″−C2″ bonds. The rotamers about the C2″−C3″ bond are designated 1, 2, and 3, while the rotamers about the C1″−C2″ bond are denoted as 4, 5, and 6.

cific assignments, we determine the relative population of the various rotamers about the C1″−C2″ and C2″−C3″ bonds of crotonyl-CoA (Cr-CoA) using three-bond proton to carbon coupling constants and ROE intensities. We also examine the database of protein-bound CoA structures and compare the structure(s) of CoA in protein−CoA complexes with the conformational preferences of free CoA. We report an interesting correlation between the conformation of free CoA and the conformation of CoA in protein−CoA complexes.

Material and Methods

Chemicals. The lithium salt of CoA was purchased from Sigma Chemical Co. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Labs.

Preparation of Crotonyl-CoA and Hexadienoyl-CoA. Cr-CoA was synthesized by mixing an excess of crotonic anhydride with 75 mg of the lithium salt of CoA in 2 mL of 50 mM $NaHCO_3$/50 mM Tris pH 8 buffer as described in the procedure of Steinman and Hill.[11] The reaction was followed by removing small aliquots of the mixture and using 5,5′-dithiobis(2-nitrobenzoic acid) to test for the presence of free thiols.[12] Cr-CoA was purified by reversed-phase HPLC using a Phenomenex Primsphere 5 C18-HC column. The gradient used was 0−75% buffer B in 40 min (buffer A, 0.2 M ammonium acetate and 1.75% CH_3CN in H_2O, buffer B, pure CH_3CN). The retention time was 15 min with a 8 mL/min flow rate. The sample was lyophilized overnight, redissolved in H_2O, and relyophilized two times. hexadienoyl-CoA (HD-CoA) was prepared as previously described.[13]

NMR Spectroscopy. NMR spectra were acquired at the SUNY Stony Brook NMR center on a Bruker AMX-600 spectrometer and on Varian Inova 500 and 600 MHz spectrometers. Samples used for the assignments of the exchangeable ^{1}H resonances were made up in 90% H_2O/10% D_2O at pH 4.3. The concentration of the sample was approximately 5 mM. Samples used for the heteronuclear NMR experiments were made up in D_2O containing 2% CD_3CN and 0.3 M KCl. The pD was 8.4 after correcting for isotope effects. These conditions were chosen to obtain the maximum concentration of sample for the heteronuclear experiments. There was no sign of aggregation under these conditions. A 60 mM sample was used for the heteronuclear experiments. All experiments were performed at 25 °C. The water resonance was attenuated by presaturation during the 1.5-s relaxation delay. ^{1}H resonance assignments were obtained using double-quantum-filtered correlated spectroscopy (DQF-COSY)[14,15] and rotating frame Overhauser effect spectroscopy (ROESY).[16] Quadrature detection was achieved by the use of time proportional phase incrementation (TPPI) except where indicated.[17] Data matrices were 512 real by 4096 complex for the DQF-COSY experiment and 512 real by 2048 complex for the ROESY experiment. The ROESY mixing time was 250 ms. The ^{13}C resonances from carbons with a directly attached proton were assigned using heteronuclear multiple-quantum-coherence (HMQC) spectroscopy.[18,19] The ^{13}C resonances from carbons without a directly attached proton were assigned using heteronuclear multiple-bond correlation (HMBC) spectroscopy. The pulse sequence of Bax and Summers was used.[20] For both the HMQC and the HMBC experiments, quadrature detection was achieved using the method of States.[21] The acquisition parameters for the HMQC experiment were as follows: data size 88 complex (^{13}C) by 4096 complex (^{1}H); spectral width 6000.6 Hz for ^{1}H and 37 709 Hz for ^{13}C. A total of 64 scans were collected for each *t*1 increment. A BIRD pulse and a 300-ms delay time were used to suppress protons that are not coupled to ^{13}C. ^{13}C decoupling during ^{1}H acquisition was achieved by the WALTZ decoupling scheme. Two HMBC spectra were acquired: one with a wide ^{1}H spectral width for assignment purposes and the other with a narrow ^{1}H spectral width which was used to measured the three-bond ^{1}H−^{13}C coupling constants. The acquisition parameters for the experiment recorded for the assignments were as follows: data size 128 complex (^{13}C) by 4096 complex (^{1}H); spectral width 6000.6 Hz for ^{1}H and 37 709

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Hz for ^{13}C. 96 scans were accumulated per *t*1 increment. The delay time for the development of the multiple-bond magnetization was 55 ms. The acquisition parameters for the HMBC experiment recorded for the purpose of measuring three-bond $^{1}H-^{13}C$ coupling constants were as follows: data size 256 complex (^{13}C) by 8192 complex (^{1}H); spectral width 2000.8 Hz for ^{1}H and 37 709 Hz for ^{13}C. A total of 128 scans were accumulated per *t*1 increment. The delay time for the development of the multiple-bond magnetization was 55 ms. The digital resolution in the proton dimension of the HMBC experiment was enhanced by the use of a narrow spectral width centered on the region of interest. As a consequence, some peaks outside of this region were aliased. The final digital resolution was 0.25 Hz. All data were processed using the program FELIX on SGI work stations. The data sets were zero-filled once in each dimension and multiplied by a shifted sine bell window function. Each FID in the ^{13}C dimension was extended by 20% using linear prediction. The first *t*1 point was multiplied by 0.5 to reduce *t*1 noise. ^{1}H and ^{13}C chemical shifts were internally referenced to 3-(trimethylsilyl)propionic-2,2,3,3,-d_4 acid sodium salt (TSP) at 0.0 ppm.

Transferred NOE spectra of HD-CoA were recorded as previously described.[13]

Simulation of Long-Range $^{3}J_{CH}$ Coupling Constants. Long-range $^{3}J_{CH}$ coupling constants were measured using an HMBC experiment. Some of the cross-peaks were distorted due to the additional splitting caused by homonuclear proton−proton couplings and in some cases by coupling to ^{31}P. $^{3}J_{CH}$ coupling constants were extracted from these peaks using the method proposed by Keeler and co-workers.[22,23] This method is based upon the comparison of a spectrum reconstructed from a 1D ^{1}H spectrum (the reference spectrum) to the cross section of the experimental spectrum (the target spectrum). The reference spectrum was obtained by extracting the specific peak of interest from a 1D proton spectrum recorded with the same delay time used in the HMBC experiment. The reference spectrum was acquired immediately after the HMBC experiment to ensure that the sample conditions were identical. The target spectrum was obtained by extracting the same proton peak from the ^{13}C vector of interest in the HMBC experiment. The intensity of the reference peak was, in general, different from the intensity of the same proton peak in the target spectrum; consequently, a global scale factor was applied. The reference spectrum was multiplied by a scaling factor and left-shifted by half of the trial $^{3}J_{CH}$ coupling constant. Another identically scaled reference spectrum was right-shifted by half of the trial $^{3}J_{CH}$ coupling constant and inverted. The two spectra were then coadded to generate the reconstructed spectrum. The sum of the absolute value of the residuals as a function of trial $^{3}J_{CH}$ coupling constant was minimized. The simulations were performed using an in-house program. The program was tested using the cross-peaks between the H3″ proton and the C10″ and the C11″ carbons. These $^{3}J_{CH}$ coupling constants can be measured to very high accuracy using the method of Kim and Prestegard since their line shapes are not complicated by proton−proton couplings.[24] A well-defined minimum of the residual was found at a single value of the trial coupling constant, and this value agrees very well with the value measured directly from the antiphase splitting, indicating that the simulation program gives accurate results.

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Results and Discussion

^{1}H NMR Assignments of Cr-CoA. A schematic drawing of Cr-CoA is shown in Figure 1 and indicates the numbering system used. The nomenclature was chosen to match that used in our transferred NOE study of HD-CoA.[13] The proton assignments were completed using DQF−COSY and ROESY experiments. The H5′ protons are the only two degenerate protons which are *J*-coupled to a single proton. They give rise to a distinctive pattern of cross-peaks, enabling their identification. The resonances of the ribose protons were readily assigned on the basis of the observed *J*-coupling connectivities using the H5′ protons as a starting point. The two adenine ring protons were assigned using the ROESY spectrum. The peak at 8.54 ppm was assigned to the H8 proton on the basis of the substantial ROEs to the H2′ and H1′ ribose protons. There are no ROE peaks between the proton resonance at 8.24 ppm and any of the ribose protons, indicating that this resonance is due to the H2 proton.

The assignment of the pantetheine protons was straightforward. The H3″ proton resonance is a singlet. The two H1″ protons were identified by their *J*-coupling to the phosphorus. They were also the only pair of protons that had homonuclear *J*-couplings only to themselves. The resonances due to the two C2″ methyl groups were easily recognized by their characteristic chemical shifts and intensities and were confirmed by their ROEs to the H1″ and the H3″ protons. The N1″ proton, the H5″ protons, and the H6″ protons give rise to a characteristic pattern of connectivities in the DQF-COSY spectrum. The N2″ proton, the H8″ protons, and the H9″ protons give rise to a similar pattern. The two spin systems were distinguished by the intense ROE from the N1″ proton to the H3″ proton. With the exception of the two H1″ protons, all of the other pairs of methylene protons are degenerate. The assignment of the diastereotopic H1″ protons is described below.

The resonances of the crotonyl protons were readily assigned on the basis of their *J*-coupling connectivity and peak intensities. The measured coupling constant between the Ha and the Hb protons across the double bond is 15.5 Hz, consistent with a trans configuration. The complete ^{1}H assignments are listed in Table 1. The ^{1}H assignments of the CoA portion agree with those made by Anderson and co-workers with one minor exception. There are two ribose protons that resonate near 4.8 ppm; Anderson assigned the most upfield of these to the 2′ position while we assign it to the 3′. The differences in the chemical shifts are very small and likely reflect the different sample conditions used.

^{13}C NMR Assignments of Cr-CoA. The ^{13}C resonances of Cr-CoA were assigned using a combination of 2D HMQC and HMBC spectra. The HMQC spectrum is shown in Figure 2. All the resonances of proton-attached carbons were readily assigned based on the one-bond $^{1}H-^{13}C$ correlation cross-peaks. The assignments were confirmed by the two-bond and three-bond $^{1}H-^{13}C$ correlation cross-peaks in the HMBC spectrum. The chemical shifts of the C8″ and C9″ carbons of the pantetheine group differ from those reported by Anderson and co-workers. This is not surprising since they were studying free CoA.

As expected, there are no cross-peaks from the carbonyl carbons or from the other nonproton-attached carbons in the HMQC spectrum. The ^{13}C resonances of these carbons were assigned based on the two-bond and the three-bond $^{1}H-^{13}C$ correlation cross-peaks in the HMBC spectrum. The HMBC spectrum of Cr-CoA is shown in Figure 3. The ^{13}C resonance at 41.55 ppm has HMBC cross-peaks to the two H1″ protons,

Table 1. ^{1}H and ^{13}C Assignments of Crotonyl-CoA in D_2O at pD 8.4, 25 °C

^{1}H	δ (ppm)	^{13}C	δ(ppm)
2	8.24	2	155.90
		4	152.05
		5	121.36
		6	158.40
8	8.54	8	142.90
NH_2	6.84[a]		
1′	6.16	1′	89.53
2′	4.82	2′	77.25
3′	4.77	3′	76.64
4′	4.58	4′	86.87
5′	4.25	5′	68.67
1″d	3.84 (*pro-R*)	1″	74.83
1″u	3.56 (*pro-S*)		
2″		2″	41.55
3″	4.02	3″	77.15
		4″	177.30
1″N	8.03[a]		
5″	3.46	5″	38.40
6″	2.44	6″	38.20
7″		7″	176.50
2″N	8.17[a]		
8″	3.34	8″	41.63
9″	3.02	9″	30.90
10″	0.89 (*pro-R*)	10″	23.68
11″	0.75 (*pro-S*)	11″	21.00
		1[c]	196.64
2[c]	6.19	2[c]	132.10
3[c]	6.95	3[c]	147.27
4[c]	1.87	4[c]	20.40

[a] Measured in 90% H_2O/10% D_2O, pH 4.3, 25 °C.

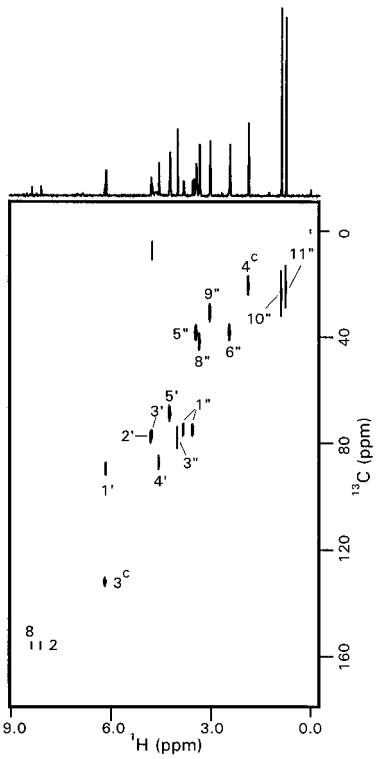

Figure 2. The $^{1}H-^{13}C$ HMQC spectrum of Cr-CoA recorded in D_2O at pD 8.4, 25 °C. The cross-peak between the Hb proton and the C2[c]-carbon is visible at lower contour levels. The projection of the ^{1}H dimension is shown at the top of the figure.

as well as to the protons of the two C2″ methyl groups and to the H3″ proton, indicating that this signal arises from the C2″ carbon. The resonance at 177.3 ppm has HMBC cross-peaks to the H3″ and the H5″ proton resonances, indicating that this

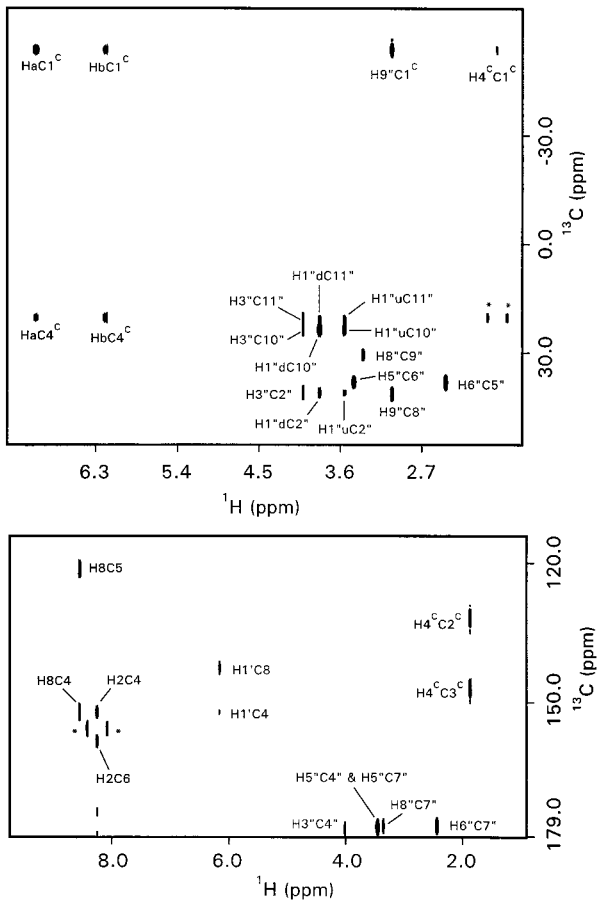

Figure 3. The $^{1}H-^{13}C$ HMBC spectrum of Cr-CoA recorded in D_2O at pD 8.4, 25 °C. The upper panel shows cross-peaks to the upfield ^{13}C resonances, and the lower panel shows cross-peaks to the downfield ^{13}C resonances. The peaks labeled with an asterisk result from incomplete suppression of one-bond correlations.

signal is due to the C4″ carbonyl carbon. The resonance at 176.5 ppm has HMBC cross peaks to the H5″, the H6″, and the H8″ protons. The pattern of correlations indicates that this signal is from the C7″ carbonyl carbon. The very downfield carbon resonance at 196.64 ppm has HMBC cross-peaks to the crotonyl Ha and Hb proton resonances as well as to the H9″ proton resonances, indicating that this is the C1[c] carbonyl carbon. Note that this peak is aliased in the spectrum and appears at an apparent shift of −53.43 ppm.

All of the carbons on the aromatic adenine ring resonate in the chemical shift range of 121 to 158 ppm. The resonances of the C2 and the C8 carbons were easily assigned based on the one-bond $^{13}C-^{1}H$ correlations to their attached protons. The resonance at 152.05 ppm is assigned to the C4 carbon since it is the only ^{13}C resonance that has HMBC cross-peaks to both the H8 and the H2 protons. The only HMBC cross-peak from the ^{13}C resonance at 121.36 ppm is to the H8 proton resonance. This demonstrates that the resonance at 121.36 ppm is from the C5 carbon. The ^{13}C resonance at 158.4 ppm has only one HMBC cross-peak. This peak is to the H2 proton, indicating that this ^{13}C resonance is due to the C6 carbon. The complete ^{13}C assignments of Cr-CoA are listed in Table 1.

Stereospecific Assignment of the C2″ Methyl Resonances. The two C2″ methyl resonances can be stereospecifically assigned by making use of data collected in the course of our transferred NOE (TRNOE) experiments on the complex of hexadienoyl-CoA (HD-CoA) with the enzyme enoyl-CoA

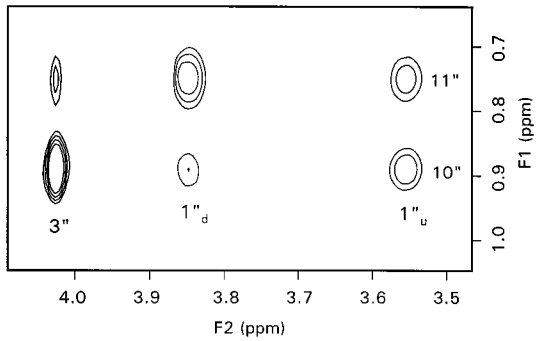

Figure 4. A portion of the transferred NOE spectrum (50-ms mixing time) of HD-CoA in the presence of enoyl-CoA hydratase. The subscripts u and d refer to the upfield and downfield 1″ protons.

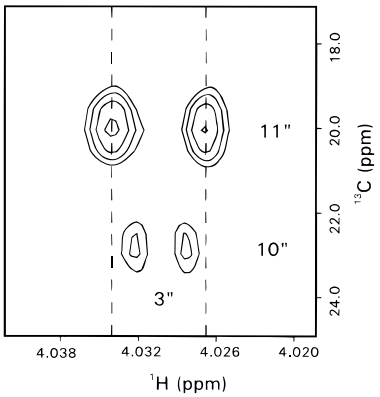

Figure 5. A portion of the HMBC spectrum of Cr-CoA showing the cross-peaks between the H3″ proton and the 2″ methyl carbons.

hydratase.[13] The stereospecific assignments of the CoA portion of HD-CoA will be identical to those of Cr-CoA since the two molecules differ only in the group that is linked to CoA and both molecules are largely unstructured in solution. It is also worth noting that the measured relative intensities of the cross-peaks in the ROESY spectrum of free HD-CoA are in excellent agreement with the values expected on the basis of the conformational analysis of Cr-CoA. In the TRNOE experiments, the observed NOEs appear at the chemical shift positions of free HD-CoA but the observed NOEs reflect *only* the bound conformation of the molecule. This is due to the fact that no NOEs are observed for free HD-CoA under the conditions of these experiments. We were able to demonstrate that the CoA portion of the bound HD-CoA is strikingly similar to the conformation of the CoA portion of 4-hydroxybenzoyl-CoA bound to the enzyme 4-chlorobenzoyl-CoA dehalogenase.[13] Wierenga and co-workers have recently solved the structures of complexes of enoyl-CoA hydratase with acetoacetyl-CoA (AcAc-CoA)[25] and with octanoyl-CoA.[26] The structure of the CoA portion of the two bound analogues is the same as the structure of the CoA portion of HD-CoA bound to enoyl-CoA hydratase. In all three of these structures, the H3″ proton is considerably closer to the *pro-R* methyl than to the *pro-S* methyl. For example, in the bound state of AcAc-CoA, the H3″ proton is 3.0 Å away from the pseudoatom of the *pro-R* methyl and 3.8 Å away from the pseudoatom of the *pro-S* methyl. This difference should give rise to clearly measurable differences in the intensity of the transferred NOEs between the H3″ proton and the diastereotopic methyl groups. This is precisely what we observe in our experiments. The H3″ proton is clearly closer to the 10″ methyl than to the 11″ methyl group as indicated by its much stronger NOE cross-peak to the 10″ methyl resonance than to the 11″ methyl resonance (Figure 4). This indicates that the 10″ methyl is the *pro-R* methyl group and the 11″ methyl is the *pro-S* methyl group.

Analysis of the C2″−C3″ Rotamers. The relative population of the C2″−C3″ rotamers can be estimated using the three-bond proton−carbon coupling constants from the H3″ proton to the C2″ methyls. There are no homonuclear $^1H-^1H$ scalar coupling partners for the H3″ proton, and hence, the line shapes of the HMBC cross-peaks that involve this proton are not distorted. Therefore, the $^3J_{HC}$ coupling constants between the H3″ proton and each of the two C2″ methyl carbons can be directly measured from the antiphase splitting of the HMBC cross-peak. The section of the HMBC spectrum containing these cross-peaks is shown in Figure 5. The antiphase splitting of the cross-peak between the H3″ proton and the 11″ methyl carbon is clearly larger than the splitting of the H3″ proton to the 10″ methyl carbon cross-peak, indicating that the H3″ to C11″ $^3J_{HC}$ coupling constant is larger. The $^3J_{HC}$ coupling constant between the H3″ proton and the 11″ carbon (the *pro-S* methyl) is 4.4 ± 0.25 Hz, and the coupling constant between the H3″ proton and the 10″ carbon (the *pro-R* methyl) is 2.1 ± 0.25 Hz.

Values of $^3J_{HC}$ coupling constants as a function of the dihedral angle have been calculated by Wasylishen and Schaefer.[27] The expected $^3J_{CH}$ coupling constant for a ±60° dihedral angle is 2.0 Hz and is 8.8 Hz for a 180° dihedral angle. Using these theoretical coupling constants, the population of the three low-energy rotamers about the C1″−C2″−C3″−C4″ dihedral angle can be calculated.[28] Newman projections of the three separate noneclipsed rotamers for the C1″−C2″−C3″−C4″ torsion angle and for the 0−C1″−C2″−C3″ torsion angle are shown in Figure 1B. The numbering of the rotamers corresponds to that used by Anderson and co-workers.[7] The calculated populations are 36% for rotamer 1, 62% for rotamer 2, and 2% for rotamer 3. The calculated populations will depend on the exact choice of the values of the $^3J_{CH}$ coupling constants for the anti and for the gauche conformations. Nevertheless, altering these values by ±10% only changes the calculated populations by 5%. Hansen and co-workers proposed a different set of coupling constants. They suggested a value of 10.6 Hz for the anti conformation and 1 Hz for the gauche conformation.[29] Using these values, the calculated rotamer populations are 35% for rotamer 1, 53% for rotamer 2, and 12% for rotamer 3. The calculated rotamer populations are also sensitive to any error in the measurement of the *J*-couplings. Varying the coupling constants by 0.3 Hz alters the populations by less than 5%, and allowing the coupling constants to vary by 0.5 Hz leads to a change in the populations of only 8%. The calculated populations of the three rotamers agrees very well with the intensity ratio of the ROE peaks between the H3″ proton and each of the methyl groups measured for free Cr-CoA. The ratio of the intensity of the ROE peak between the H3″ and the 10″ methyl protons to the intensity of the ROE peak between the H3″ and the 11″ methyl protons is 1.8. The ROE ratio calculated on the basis of the populations of the three rotamers is 1.7. The measured $^3J_{HC}$ coupling constants and the ROE ratio unambiguously rule out any substantial population of rotamer 3.

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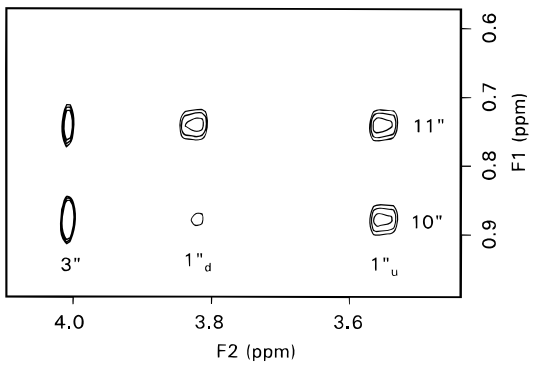

Figure 6. A portion of the ROESY spectrum of Cr-CoA recorded in D_2O at pD 8.4, 25 °C, showing the cross-peaks from the 10″ and 11″ methyl resonance to the H1″u, H1″d, and H3″ protons.

Previous studies of free CoA have assumed that rotamer 2 is not significantly populated. This assumption is based on the observation of unequal NOE intensities between the H3″ proton and the two C2″ methyl groups. However, unequal intensities can be observed even if rotamer 2 is the most populated rotamer providing that the populations of rotamer 1 and rotamer 3 are unequal. If rotamer 1 was exclusively populated, then the predicted ratio of NOE intensities would be 4.3 to 1. The measured $^3J_{CH}$ coupling constants are critical for accurately determining the populations of the rotamers.

Stereospecific Assignment of the 1″ Protons and Analysis of the C1″−C2″ Rotamers. The 1″ protons can be stereospecifically assigned by three methods. Transferred NOE data can be combined with analysis of the known crystal structures to assign these resonances in the same way that was used to assign the C2″ methyls. Alternatively, the $^3J_{CH}$ coupling constants between the 1″ protons and the two methyl groups at C2″ can be used to distinguish between the two diastereotopic 1″ protons. The ROE intensities measured for free Cr-CoA can also be used. Since the two diastereotopic 2″ methyl groups have been assigned, the relative intensities of the ROE from each of these groups to the two diastereotopic 1″ protons can be used to stereospecifically assign the 1″ protons. A portion of the ROESY spectrum is shown in Figure 6. It is clear that the downfield 1″ proton (referred to as H1″d) is closer to the 11″ methyl than to the 10″ methyl. The ratio of the intensities of the H1″d−11″methyl ROE cross-peak to the H1″d−10″methyl ROE cross-peak is 2.5. In contrast, the ROEs from the upfield 1″ proton (referred to as H1″u) to each of the two 2″ methyl groups are of equal intensity. This pattern of intensities indicates that the dominant rotamer about the C1″−C2″ bond is rotamer 4. The H1″d proton is thus the *pro-R* proton and the H1″u is the *pro-S* proton.

The line shape of the HMBC cross-peaks between the 1″ protons and the methyl groups at C2″ is distorted by homonuclear couplings as well as by coupling to phosphorus; therefore, simulations are required to extract the values of $^3J_{CH}$ coupling constants.[22,23] The three-bond H1″d−C10″ and H1″d−C11″ coupling constants were found to be 5 ± 0.25 and 2.3 ± 0.25 Hz. A plot of the reconstructed spectrum and the target spectrum is shown in Figure 7A for the coupling pair of the H1″d proton and the C10″ carbon. A plot of the sum of the absolute value of the residuals versus the trial *J*-coupling constant is shown in Figure 7B. The measured coupling constant between the H1″d proton and the 10″ methyl carbon (*pro-R*) indicates that the H1″d proton is anti to the 10″ methyl carbon. The smaller coupling constant between the H1″d proton and the 11″ methyl (*pro-S*) suggests that the H1″d proton is

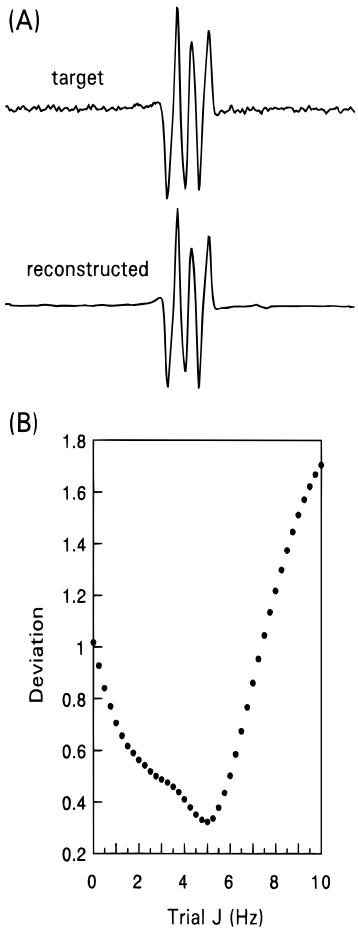

Figure 7. Determination of $^3J_{CH}$ coupling constants via line shape simulations. (A) A comparison of the reconstructed spectrum and the target spectrum for the ^{13}C row vector of the HMBC cross-peak between the H1″d proton and the 10″ carbon. (B) The deviation between the reconstructed spectrum and the target spectrum plotted versus the trial value of $^3J_{CH}$.

gauche to the 11″ methyl carbon. The coupling constant between the H1″u proton and the 10″ methyl carbon is 2.75 ± 0.25 Hz while the coupling between the H1″u proton and the 11″ methyl carbon is 2.3 ± 0.25 Hz. These values of the *J*-coupling constants indicate that the H1″u proton is gauche to both of the two methyl groups. The two sets of measured $^3J_{CH}$ coupling constants predict slightly different rotamer distributions, but both sets predict that rotamer 6 is not populated, and both predict a significant population of rotamer 4. In rotamer 4, the H1″d proton is the *pro-R* proton and the H1″u proton is the *pro-S* proton. These assignments are consistent with the results of the ROESY experiments. The calculated population of rotamer 4 is 64 ± 20%, the calculated population of rotamer 5 is 28 ± 24%, and the calculated population of rotamer 6 is only 8 ± 3%. The calculated populations are the average of the values determined using the two separate sets of measured coupling constants. The uncertainties reflect the maximum difference between the average value and the individual values. Analysis of the transferred NOE data also leads to the same stereospecific assignments. The low population of rotamer 6 is consistent with the earlier work of Anderson and co-workers.[7]

It is interesting to note that examples of both rotamer 4 and rotamer 5 are found in the database of CoA−protein complexes but there no examples of a bound structure adopting rotamer 6. In both rotamer 4 and rotamer 5, the C3″ hydroxyl group can potentially form a hydrogen bond with one of the oxygens of

the pyrophosphate moiety provided that the conformation about the C2″−C3″ bond corresponds to rotamer 1. This interaction is not possible in rotamer 6. If the C2″−C3″ bond corresponds to rotamer 2, then the C3″ hydroxyl group can potentially interact with the pyrophosphate region if the rotamer 4 is populated but not if rotamer 6 is populated. Local interactions between the C3″ hydroxyl and the pyrophosphate have also been observed in computational studies.[7,30]

Conformation about the O−C1″ Bond. The three-bond $^3J_{HP}$ coupling constants between each of the 1″ protons and the β-phosphorus can be used to define the conformation about the O−C1″ bond. The two coupling constants are both found to be 5 Hz. The expected $^3J_{HP}$ coupling constant for a 180° dihedral angle is 23.0 Hz, and the expected value for a ± 60° dihedral angle is 2.1 Hz.[31] The measured coupling constants correspond to a population distribution of 72% trans and 14% each of the two gauche conformations between the β-phosphorus and the C2″ carbon. This result is in agreement with previous studies of CoA and CoA derivatives.[2,7]

Comparison with CoA−Protein Crystal Structures. It is of interest to examine the conformation of CoA in CoA−protein complexes. In general, CoA is observed to adopt a wide range of bound conformations, but there do appear to be certain common features. Anderson and co-workers published a summary of the bound conformation of CoA in a total of nine X-ray structures derived from five separate proteins.[7] They noted that rotamer 1 was preferred, although there were two structures in which rotamer 3 was populated. Since that time a number of additional X-ray structures have appeared and the size of the database has doubled. The new structures include the CoA:succinyl-CoA synthetase complex (pdb code, 1scu), the α-flouroamidocarboxymethyldethia-CoA:citrate synthase complex (1csr), the α-flouro-carboxymethyldethia-CoA:citrate synthase complex (1css), the acetoacetyl-CoA:butyryl-CoA dehydrogenase complex (1buc), the 4-hydroxybenzoyl-CoA:4-chlorobenzoyl-CoA dehalogenase complex (1nzy), the acetoacetyl-CoA:enoyl-CoA hydratase complex (1dub), and the octanoyl-CoA:enoyl-CoA hydratase complex (2dub). In all of these structures, the bound CoA derivatives adopt a conformation about the C2″−C3″ bond corresponding to rotamer 1. There are no additional examples of rotamer 3. Anderson and co-workers also examined the conformation about the C1″−C2″ bond, and noted that rotamer 6 was never populated. Our analysis of the newly solved CoA−protein complexes indicates that there are no occurrences of this rotamer. There is only one example of a bound CoA derivative adopting rotamer 5 (1buc). All other CoA derivatives exclusively adopt rotamer 4. Thus it appears that there are strong local conformational preferences in bound CoA structures and that these reflect, in part, the conformational preferences of free CoA. In particular, rotamers 3 and 6 are not populated in free CoA. There are no examples of a bound CoA derivative adopting rotamer 6 and only two out of the known 16 structures adopt rotamer 3.

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Conclusions

The complete ^{1}H and ^{13}C assignment of Cr-CoA have been achieved. The assignments have allowed a detailed and quantitative analysis of the conformational tendencies of CoA in solution. A search of the protein database has revealed a correlation between the conformational properties of free CoA and the structure of CoA in protein−CoA complexes. The stereospecific assignments will prove useful in other transferred NOE studies.

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