Characterization of the Carboxylate Delivery Module of Transcarboxylase: Following Spontaneous Decarboxylation of the 1.3S-CO_2^- Subunit by NMR and FTIR Spectroscopies†

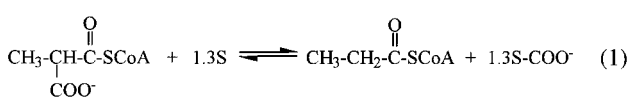
Rosa E. Rivera-Hainaj,‡,# Marianne Pusztai-Carey,‡ D. Venkat Reddy,⊥,§ Kiattawee Choowongkomon,⊥ Frank D. Sönnichsen,⊥ and Paul R. Carey*,‡

Department of Biochemistry and Department of Physiology and Biophysics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4935

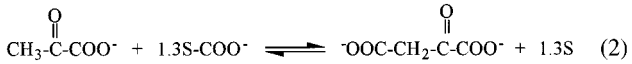
Received August 9, 2001; Revised Manuscript Received December 5, 2001

ABSTRACT: Transcarboxylase (TC) is a multisubunit enzyme that catalyzes the transfer of a carboxylate group from methylmalonyl-CoA (MMCoA) to pyruvate. The CO_2^- group is shuttled between the MMCoA and pyruvate binding sites by a biotin cofactor, covalently linked to the 1.3S subunit. Fully carboxylated 1.3S can be prepared in vitro using 1.3S, MMCoA, and catalytic amounts of the TC's MMCoA-binding subunit. The 1.3S−CO_2^- intermediate decarboxylates spontaneously over a period of hours, and this process was characterized by 1D and 2D NMR and FTIR spectroscopies. The NMR data yielded a first-order kinetic constant of 1.4×10^{-3} min^{-1} for the spontaneous decarboxylation. This rate was calculated from the 1D NMR spectrum by measuring the reappearance of biotin's ureido NH protons and the disappearance of peaks at 6.99 and 7.67 ppm assigned to Asn-8 and/or Asn-24 from the 1.3S's N-terminus. The latter peaks are absent in the 1D spectrum of non-carboxylated 1.3S due to exchange between two or more conformations within the N-terminus causing line broadening. It is proposed that interactions between the biotin-CO_2^- and the N-terminal amino acids perturb this conformational equilibrium causing some N-terminal residues to appear in the 1D NMR spectrum of the carboxylated form. Further details are apparent from a comparison of the 2D spectra of the 1.3S−CO_2^- and 1.3S proteins, where carboxylation causes several peaks from the C-terminal half to shift as well as the appearance of resonances due to some residues located at the N-terminal half of the protein. FTIR difference spectra were used also to follow spontaneous decarboxylation of the 1.3S−CO_2^-. For the carboxylated 1.3S, the difference spectra provided the vibrational signature of the CO_2^- on the biotin ring. A doublet was identified at 1695 and 1699 cm^{-1} that increased in intensity with increasing *t*. This is assigned to an antisymmetric stretching vibration of the CO_2^- group bound to biotin on the 1.3S protein. Its position and profile provide further evidence for interactions occurring between the biotin-CO_2^- group and the 1.3S protein. These studies demonstrate the highly mobile, "poised" nature of the 1.3S protein engineered for its role as a CO_2^- translocator.

Transcarboxylase (TC),[1] a biotin-containing enzyme found in *Propionibacterium shermanii*, provides an opportunity for studying the structure and function of a large multisubunit enzyme and for exploring the mechanism of carboxylate transfer reactions that occur in a number of important mammalian enzymes. Part of TC's attraction lies in its modular nature; it consists of 30 polypeptides that belong to three different subunit classes: 1.3S, 12S, and 5S. TC can be assembled and disassembled by changing pH and salt conditions (*1*). At the interface of the 1.3S and 12S subunits, TC catalyzes the transfer of a carboxylate group from methylmalonyl-CoA (MMCoA) to the biotin cofactor linked to the 1.3S.



After translocation of the biotin-CO_2^- on the 1.3S protein to the 5S subunit active site, the carboxylate group binds to pyruvate to form oxaloacetate.



All the subunits have been cloned into and purified from *Escherichia coli*, and thus each half-reaction can be studied separately.

† The present study was supported by NIH Grant GM 18575 (R.E.R.H.), NIH Grant GM53053 (P.R.C.), and NIH Grant GM55362 (F.D.S.).

* To whom correspondence should be addressed: carey@biochemistry.cwru.edu. Tel: 216-368-0031. Fax: 216-368-3419.

‡ Department of Biochemistry, Case Western Reserve University.

⊥ Department of Physiology and Biophysics, Case Western Reserve University.

Present address: Department of Chemistry, John Carroll University, University Heights, OH 44118.

§ Present address: Department of Chemistry, University of Akron, Akron, OH 44325.

[1] Abbreviations: MMCoA, methylmalonyl coenzyme A; MCoA, malonyl coenzyme A; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared; TC, transcarboxylase; BCCP, biotin carboxy carrier protein; TFA, trifluoroacetic acid; HSQC, heteronuclear single quantum coherence spectra; DSS, 4,4-dimethyl-4-sila-pentane sulfonate.

10.1021/bi0116442 CCC: $22.00
Published on Web 01/26/2002

In the present work, we continue our analysis of the smallest subunit of TC, the "carboxylate shuttle" 1.3S (*2*−*5*). The biotinyl or 1.3S subunit is an 123-amino acid monomer ($M_r \sim 12\,600$ Da), with its biotin cofactor covalently bound to the ϵ-amino group of Lys-89, which lies in the conserved sequence Ala−Met−Lys−Met (*6*). The three-dimensional structure of the 1.3S subunit has been solved by multidimensional-NMR (*5*) and forms two distinct domains. The C-terminus (residues 51−123) is folded into a compact β-sheet sandwich with a 2-fold symmetry. A similar fold has been described for the homologous *E. coli* acetyl-CoA carboxylase BCCP (biotin-carboxy carrier protein) domain, but the 1.3S subunit lacks an extension named the "protruding thumb" found in BCCP (*5*, *7*). The C-terminus β-sheet sandwich of 1.3S provides a rigid platform to which the biotin cofactor is bound. This raises the possibility that the entire β-sandwich translocates between the 12S and 5S subunits during the reaction cycle.

The N-terminal half of the 1.3S subunit is more enigmatic. Early biochemical studies showed that the amino acids near the N-terminus of the 1.3S subunit play a role in holding the 5S and 12S subunits in place (*8*). In our NMR analysis (*3*−*5*), we described the N-terminal region of the 1.3S subunit (residues 1−50) as a tail lacking in order, due to the absence of resonances in the NMR spectra that could be assigned to N-terminal residues. However, new evidence, presented herein, suggests that the N-terminal region is partially ordered in solution. The failure to detect N-terminal residues before is ascribed to the fact that this part of the 1.3S molecule is undergoing intermediate exchange between two or more conformational states.

The enzymatic carboxylation of D-biotin involves the replacement of the N1′ ureido proton by a carboxylate group, and the structure and electronic properties of the carboxybiotin molecule have been discussed widely (*9*−*12*). The present work takes advantage of the fact that the 1.3S subunit of TC can be carboxylated in vitro in the presence of the 12S subunit, MMCoA (or malonyl-CoA). With NMR and FTIR spectroscopies, we were able to measure the rate of spontaneous decarboxylation of the 1.3S−CO_2^- intermediate. Both techniques indicate that the carboxylated biotin interacts with the N-terminal half of 1.3S, although our earlier NMR studies (*3*) found no evidence for interactions between noncarboxylated biotin and the 1.3S protein.

MATERIALS AND METHODS

Reagents. Malonyl-CoA, D_2O, avidin-monomeric agarose resin, and D-biotin were purchased from Sigma Chemical Company (St. Louis, MO). Potassium phosphate salts were purchased from Fisher Scientific (Pittsburgh, PA), and P-6 desalting columns were purchased from Biorad (Richmond, VA).

Protein Purification. The recombinant unlabeled 1.3S and the ^{15}N-labeled protein were expressed in *E. coli* and purified according to published procedures (*13*). A Vydac reverse phase C8 column (Hesperia, CA) with dimensions 2.2 cm × 25 cm was used with a 2-mL loop. The initial flow rate was 8 mL/min, and then it was reduced to 4 mL/min after 20 min. A gradient program was applied in which the solvents used were 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The protein elution was monitored at 220 nm, and the average elution times were 28 min for the apo-1.3S, and 34 min for the holo-1.3S (at 38% solvent B). The recombinant 12S subunit was expressed in *E. coli* and purified according to published procedures (*14*).

Sample Preparation. For the NMR studies of pH effect on the 1.3S subunit, a 1 mM solution of the protein was prepared in water. The pH of the solution was adjusted by adding either HCl or NaOH to obtain the desired pH's. These studies were carried in the absence of buffer, as published previously (*3*).

The 1.3S subunit (3−4 mM) was carboxylated in the presence of the 12S subunit (1 μM), MCoA (10 mM), and potassium phosphate buffer (0.5 M, pH 6.5). MCoA was used as a substrate analogue for MMCoA since MCoA lacks a chiral center on the malonyl moiety, which allows all the substrate to be utilized without the protein having to select from a racemic mixture. The K_m for MMCoA is 4.3 μM and the K_m for MCoA is 0.35 μM (*15*). The reaction mixture was incubated at room temperature for 15 min. The carboxylated 1.3S (1.3S−CO_2^-) was purified by gel filtration using a P-6 desalting column equilibrated with potassium phosphate buffer (0.1 M, pH 6.5) at 4 °C. The absorbance of the collected fractions was monitored at 220 nm with a Shimadzu UV−Vis spectrophotometer (Japan). The fractions containing carboxylated-1.3S were collected together to a final volume of 480 μL and transferred to a NMR tube, where 20 μL of D_2O was added to act as a "lock" signal. The final concentration of the carboxylated protein was about 1 mM. The pH measurements reported are the direct reading from the pH meter and are not corrected for the use of D_2O.

For the FTIR experiments, the 1.3S subunit (3−4 mM) was carboxylated in D_2O in the presence of the 12S subunit (1 μM), MCoA (10 mM), and potassium phosphate buffer (0.5 M, pH 6.5). The 1.3S protein was previously dissolved in D_2O and lyophilized extensively to exchange most of the NH's to ND's to avoid H−O−D vibrations as much as possible in the region around 1460 cm^{-1} in the FTIR spectra. The reaction mixture was incubated at room temperature for 15 min. The carboxylated-1.3S was isolated by gel filtration with a microspin P-6 desalting column equilibrated with D_2O, pH 6.5 (pH meter reading), at room temperature, with no buffer present. The D_2O pH had been adjusted to 6.5 with NaOD. A 50 μL sample was injected into a sealed FTIR cell with 0.025 mm path length and CaF_2 windows (International Crystals Laboratories, NJ).

Data Acquisition and Analysis. NMR experiments were carried out on a Varian Innova spectrometer operating at a proton frequency of 600 MHz. 1D-^{1}H NMR experiments and 2D-^{15}N−HSQC experiments were performed on freshly prepared carboxylated TC-1.3S within 30 min of its preparation and at regular intervals over the next 24 h. All data were acquired at 5 °C. 1D-^{1}H NMR spectra were typically obtained with a sweep width of 6000−7000 Hz, a repetition delay of 1.5 s, 128 to 512 scans, and an acquisition time of 2 s. Water suppression was achieved by either 1.5 s presaturation, or preferably with a watergate sequence (*16*), which significantly reduced intensity losses for the biotin-ureido ring protons due to exchange. For isotopically labeled samples, broadband heteronuclear decoupling was employed using WALTZ during the acquisition time of 80 ms. Chemical shifts were referenced to external DSS, and the

data were zero filled to 32k after employing an exponential apodization function of 2 to 5 Hz. The spontaneous decarboxylation of isotopically labeled TC samples was also followed by heteronuclear single quantum coherence spectra (HSQC), acquired with gradient coherence selection and sensitivity enhancement (*17*). The data consisted of 100 × 512 complex data points; they were weighted using shifted sinebell function, and zero filled to 512 × 1K complex data points. All processing and data analyzing were performed with Vnmr software on SGI workstations.

For the determination of kinetic data, all spectra were processed identically, expanded to the region of interest, and peak intensities/heights were measured after drift or baseline correction. 1D NMR spectra were used to measure the spontaneous decarboxylation rate at the biotin ring on 1.3S by following the increase in intensity of the peak at 6.52 ppm, that corresponds to the proton at the N1′ position on the biotin. The height of each peak at time t was measured. The height of the last point (after 23 h) was labeled I_∞ and the intensity at time I_t was subtracted from the intensity at I_∞. The $\ln(I_\infty - I_t)$ was plotted against time with the slope providing the rate of the reaction. Similarly, the 1D NMR spectra were used to calculate the rate of disappearance of the resonance at 7.67 ppm that is seen only for the carboxylated form of the 1.3S protein. The intensity of this peak, I, was measured and $\ln(I)$ was plotted against time. Exponential and linear fitting of the kinetic data gave the same rate constants. This demonstrated that linear fitting without the availability of an end-point for the NMR experiments (which was impractical due to lack of time for instrumental use) did not introduce a significant error. Exponential fitting does not require the use of an end-point.

FTIR experiments were carried out using a Bomem MB100 series spectrometer, operating at a resolution of 4 cm^{-1}, at 25 °C. Spectra were acquired over a period of 21 h, and each spectrum consisted of 64 scans and took 3.5 min to acquire. Difference spectra were calculated by subtracting the spectrum at time t from the spectrum at time t_0. For the determination of decarboxylation kinetics, all spectra were processed identically, expanded to the region near 1700 cm^{-1}, and peak intensities/heights were measured. The spontaneous decarboxylation rate at the biotin ring on 1.3S was measured by following the increase in intensity of the peak at 1698 cm^{-1} over time. The intensity of the last point (after 20 h) was labeled I_∞, and the intensity at time I_t was subtracted from the intensity at I_∞. The $\ln(I_\infty - I_t)$ was plotted against time with the slope providing the rate of the reaction.

RESULTS

Effect of pH on the Overall Conformation of the 1.3S Subunit. The structure of the 1.3S subunit has been characterized in solution by multidimensional NMR spectroscopy (*3*−*5*). It consists of two domains: the C-terminal region (residues 51−123) is folded into a compact β-sandwich, while the N-terminal region was characterized as being disordered in the isolated 1.3S subunit. Here we present new evidence for the 1.3S subunit that causes us to reevaluate and refine the earlier conclusion regarding the N-terminus.

In previous studies (*4*, *5*), that were primarily based on heteronuclear ^{15}N-edited techniques, resonances originating in the first 23 N-terminal residues of the 1.3S protein were not present; an observation that was attributed to conformational flexibility and rapid exchange with water. To assess the properties of this region, we therefore decided to utilize the somewhat unusual amino acid distribution of the 1.3S subunit (PRI code: P02904): there are no tryptophan residues, and all the aromatic residues are located in the N-terminal half of the sequence (*18*). One-dimensional ^1H NMR spectra in D_2O, under conditions in which all other nonaromatic resonances that potentially occur in this frequency range are exchanged and not observable, were expected to show sharp resonances at the ring protons of these three residues only. At neutral pH the resonances belonging to these residues are broad and overlapping features (*4*, *5*) (Figure 1). However, sharp features due to Tyr-12, His-22, and Phe-31 side chains do appear at low or high pH. At pH 2.9, characteristic phenylalanine resonances appear at 7.30, 7.34, and 7.39 ppm. A similar behavior is observed when the pH is increased to 11.0. Similarly, two intense resonance lines appear at 7.15 and 6.86 ppm at pH 2.4, and at 7.0 and 6.6 ppm at pH 11.0. These frequencies are characteristic of tyrosine Hδ and Hϵ protons. Other resonances appear at 8.64 and 7.3 ppm at low pH and at 7.72 and 7.0 ppm at high pH (Figure 1). These signatures are assigned to His-22, and they are essentially absent at neutral pH.

The 1.3S protein sequence has a high content of methionine and valine residues in the region of residues 1−30 (*18*). Changes in the region of the 1D NMR spectrum associated with methionine and valine occur at low and high pH that resembles the behavior of the aromatic side chains described above. The epsilon CH_3 protons of methionine have a chemical shift at 2.13 ppm when in a random coil conformation. For the 1.3S protein, a resonance at 2.1 ppm becomes an intense feature upon going from neutral to basic pH (Figure 2). The same behavior is observed when changing from neutral to acidic pH (data not shown). Further, intense resonances appear around 0.9 ppm (Figure 2), about the random coil chemical shift of valine γ-methyl groups. This resonance can be attributed to valine 5, 7, 14, 16, or 18. The γ-CH_3 protons of valine have chemical shifts of 0.94 and 0.97 ppm when free in solution. An intense resonance peak appears around 0.9 ppm upon changing from neutral to basic pH (Figure 2). All of the above observations detecting aromatic, methionine, and valine resonances in the 1D spectrum of the 1.3S subunit strongly suggest that the N-terminus of the protein undergoes a transition into a random coil or unordered conformation below pH ∼ 3.5 and above pH ∼ 9.0. Previous ^1H-^{15}N HSQC and ^1H-^{15}N correlation experiments showed that below pH 3.0 some unfolding of the protein occurs, which is characteristic of acid-driven protein unfolding. In the range between pH 3.5 and 6.4, no unfolding was observed. At the same time, no measurable unfolding was detected above pH 11.0 (data not shown). At neutral pH, broad lines and the absence of distinct resonances from residues in the N-terminus in the 1D or 2D spectra must imply that the N-terminus is in two or more conformational states and that exchange between those states is occurring on the NMR chemical shift time scale. This exchange leads to broadening of the NMR resonances and accounts for their absence in the 1D and 2D data sets. Upon changing to acidic or basic conditions, the N-terminus does

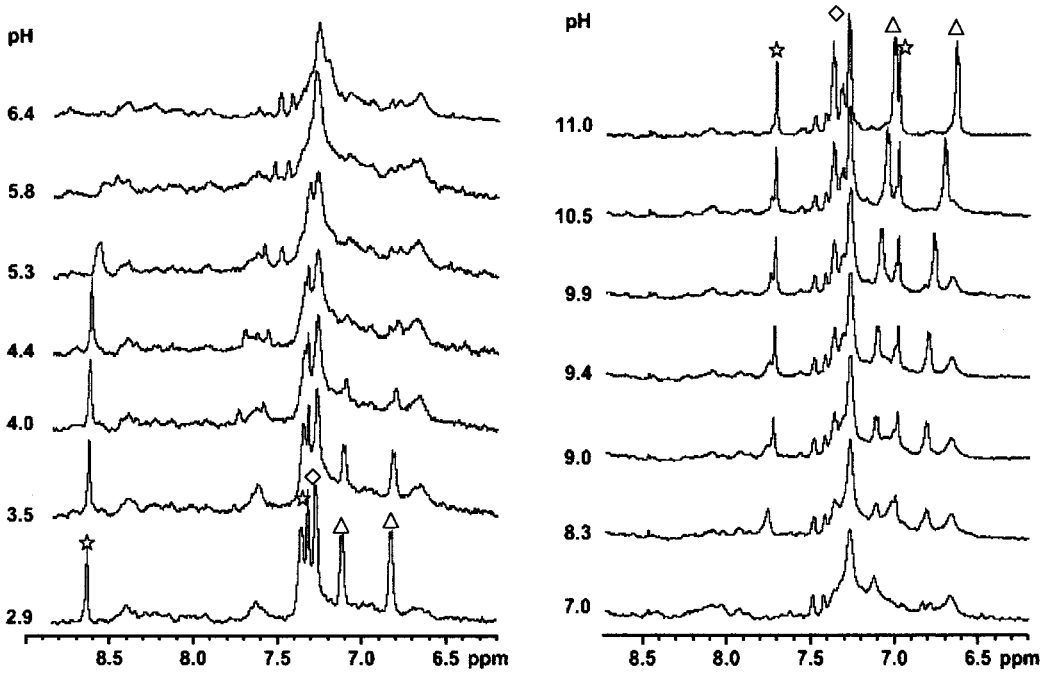


FIGURE 1: The 6.4 to 9.0 ppm region of the 1D-^{1}H NMR spectra of 1.3S in D_2O at various pH values. Under these conditions, NH resonances have completely exchanged, and only aromatic resonances are observed. At both pH extremes, three side chains, e.g., Tyr-12 (7.15 and 6.86 at pH 2.4), His-22 (8.64 and 7.3 ppm at pH 2.4), and Phe-31 (7.30, 7.34, and 7.39 ppm at pH 2.4), give rise to peaks at the frequencies expected for random coil.

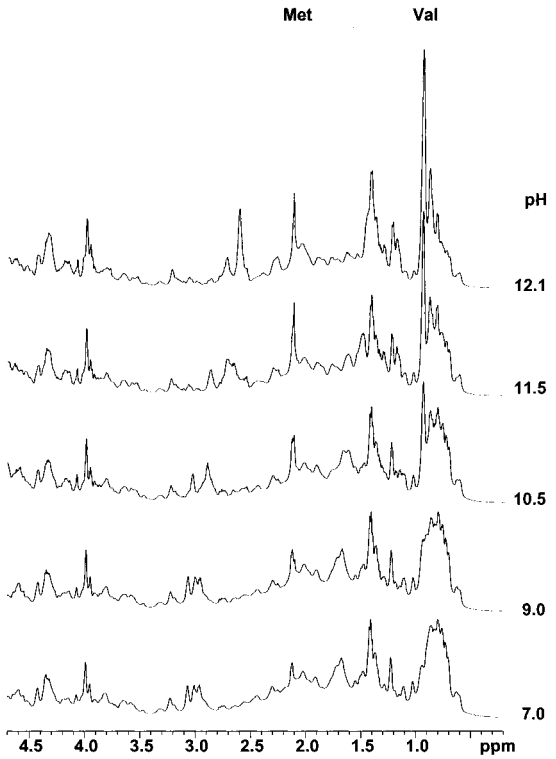


FIGURE 2: The aliphatic region of the 1D-^{1}H NMR spectra of 1.3S in D_2O taken at various alkaline pH values, where the resonances belonging to methionine and valine residues give rise to peaks at the frequencies expected for a random coil.

undergo a transition into a random coil with the appearance of many "new" NMR peaks in the 1D.

Following the Spontaneous Decarboxylation of 1.3S−COO⁻ by 1D NMR Spectroscopy. The 1.3S subunit was carboxylated in vitro, and the carboxylated protein was isolated as described in Materials and Methods. The spontaneous decarboxylation of 1.3S, in the absence of the other TC subunits, was followed by 1D and 2D NMR spectroscopies. The carboxylation of biotin in the 1.3S subunit leads to a number of changes in the 1D NMR spectrum. Prior to carboxylation, the ureido protons at the N1′ and N3′ positions of the biotin cofactor resonate in the 1D NMR spectrum at 6.5 and at 6.6 ppm, at 25 °C, respectively (*3*). Figure 3 shows a time course experiment of the 1.3S−COO⁻ spontaneous decarboxylation process. At time close to zero after the carboxylation reaction, the resonances due to the N1′ and N3′ protons at 6.5 and 6.6 ppm are essentially absent. As spontaneous decarboxylation of the biotin occurs over a period of hours, the N1′ and N3′ proton resonances increase in intensity until reaching a plateau upon full decarboxylation of the biotin. Besides these changes, new resonances are

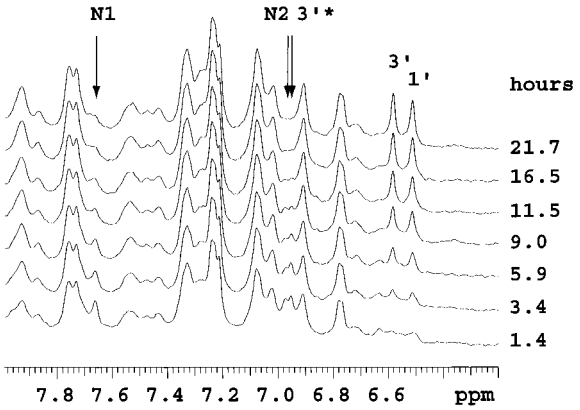


FIGURE 3: ^{1}H NMR spectra for the time course of spontaneous decarboxylation of the 1.3S−COO⁻ subunit. The spectra were taken over a period of 22 h. The 1′ and 3′ refer to the protons at positions N1′ and N3′ of the biotin ureido ring. The 3′* and N2 refer to two "new resonances" near 6.96 ppm. The N1 refers to the new resonance near 7.6 ppm. All new resonances disappear upon spontaneous decarboxylation.

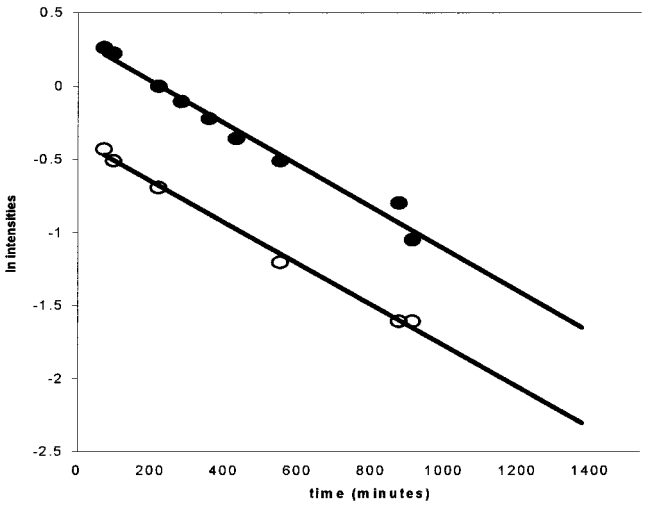


FIGURE 4: Calculated rates, from the ^{1}H NMR spectra data, for the spontaneous decarboxylation of the 1.3S−COO$^-$ complex. Open circles refer to the peak corresponding to the N1′ proton resonance (ln [$I_8 - I_x$]). Closed circles correspond to the new resonance at 7.67 ppm (ln I_8). Both lines give a decarboxylation rate of 1.4×10^{-3} min^{-1}. The R^2 value for the generated lines are 0.979 for the N1′ resonance and 0.995 for the resonance at 7.67 ppm.

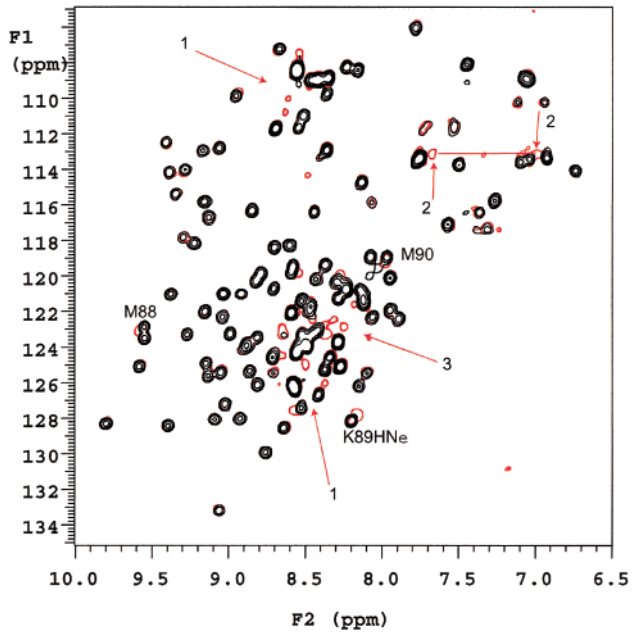


FIGURE 5: ^{1}H-^{15}N HSQC NMR spectra of the carboxylated (red) and decarboxylated (black) 1.3S subunit. Experiments were acquired in the presence of 100 mM phosphate buffer, pH 6.5, and 5 °C.

observed at 6.96, 6.99, and 7.67 ppm (3′*, N2 and N1, respectively), which decrease in intensity upon spontaneous decarboxylation of the biotin cofactor. The 7.67 ppm and one of the 6.9 ppm region resonances have been assigned to either Asn-8 or Asn-24 (discussed below). The other resonance in the 6.9 ppm region we assign to the proton at the N3′ position of the biotinyl-ureido ring. The later resonance is shifted from 6.6 ppm in the noncarboxylated biotin to the 6.9 ppm region upon carboxylation at the N1′position.

The rate of appearance of the N1′ proton peak and the rate of decrease of the new resonance at 7.67 ppm have been measured (Figure 4). The observed reaction rate at 5 °C and in 100 mM phosphate buffer, pH 6.5, is 1.4×10^{-3} min^{-1} (half-life = 495 min). This observed rate is the same for both the appearance of the N1′ proton and the disappearance of the new 7.67 ppm feature, suggesting that the reappearance of the N1′ proton and the disappearance of the 7.67 ppm "new feature" originate in the same event.

Carboxylate-Induced Structural Changes in the 1.3S Subunit. 2D NMR experiments cast light on the origin of the new resonances seen for the 1.3S−CO_2^- complex in Figure 3. The overlayed 2D-HSQC ^{1}H-^{15}N NMR spectra of the carboxylated and decarboxylated (22 h after carboxylation) 1.3S subunit are shown in Figure 5. The appearance of resonances near 6.97 and 7.66 ppm in Figures 3 corresponds to the red contours seen at the same chemical shifts in Figure 5. Both ^{15}N and ^{1}H chemical shifts, and the fact that two cross-peaks appear at identical ^{15}N-frequencies, suggest a side chain amide group as the cross-peaks origin. The 1.3S subunit contains three asparagines and three glutamines in its sequence. All three glutamines, Glu-80, Gln-114, and Gln-117, are in the C-terminal half of 1.3S protein. Of the three asparagines, two are located in the N-terminal half of 1.3S, Asn-8 and Asn-24, and one in the C-terminal half, Asn-95 (*18*, *19*). All three glutamines and Asn-95 have been previously assigned (*5*), and thus the peak at 7.66 ppm and one of the resonances near 6.96 ppm likely originate from either Asn-8 and/or Asn-24. In the non-carboxylated 1.3S, these resonances are not detected due to the presence of two or more conformations in the N-terminus that are exchanging on the NMR chemical shift time scale leading to the broadening and loss of the asparagine peaks. Carboxylation of the biotin changes the nature of the conformational space of the protein such that exchange broadening is now reduced or eliminated for some amino acids in the N-terminus. It can be seen in Figure 5 that several additional resonances appear for the carboxylated protein. Those in region "1" are likely glycine residues, and those in region "3" are likely to be from alanine residues. The N-terminal half of the 1.3S protein is rich in glycines and alanines, and the results seen in Figure 5 are consistent with some of these residues becoming visible due to the changes in conformational properties of the N-terminus upon carboxylation.

We have already seen that protonation/deprotonation of residues in the 1.3S subunit can bring about the appearance or disappearance of the aromatic amino acid side chain and other resonances in the 1D spectrum. The perturbation caused by biotin carboxylation is not the same. However, both effects point at the N-terminus to be in two or more exchanging conformational states near neutral pH that can be modified by changes in pH or by carboxylation. Interestingly, the C-terminus of the 1.3S subunit also undergoes slight changes upon carboxylation. Lys-89, to which biotin is covalently linked, and its neighbors, Met-88 and Met-90, all undergo chemical shift differences upon carboxylation (Figure 5, region '2'). Thus, although no direct interactions were detected in earlier studies between biotin and the C-terminal core, the effects of carboxylation on the biotin are transmitted to the region around Lys-89. It is plausible that the shift in resonances 88−90 is caused by the interactions between the carboxybiotin and the N-terminus.

Following the Spontaneous Decarboxylation by FTIR Difference Spectroscopy. In favorable cases, FTIR difference spectroscopy can follow changes in proteins at the level of

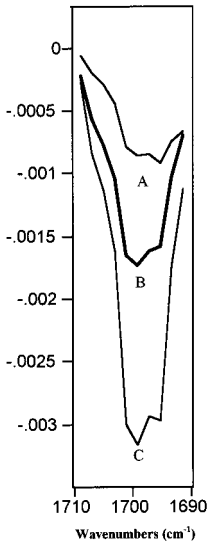


FIGURE 6: Following spontaneous decarboxylation of 1.3S−CO_2^- by infrared spectroscopy FTIR difference spectra of the 1700 cm^{-1} region as a function of time: 3 h (−), 6 h (-), and 21 h (- -).

individual chemical groups. Since the change in the FTIR spectrum is very small at the level of a single side chain, the approach needs both the sample and spectrometer to be very stable. For example, light-induced intermediates in bacteriorhodopsin films, where the sample remains fixed; provide high quality FTIR difference data (*20*). We have taken advantage of the spontaneous decarboxylation of the 1.3S−CO_2^- to detect the vibrational signature of the biotin-bound carboxylate. Small changes in the FTIR can be detected reliably because data sets can be subtracted for a sample that is changing chemically yet remains stationary in the FTIR spectrometer. Thus, we were able to detect a CO_2^- mode near 1700 cm^{-1} that had an absorbance of ~ 0.003 from a 1.5 mM sample of 1.3S−CO_2^- that had an absorbance at 1650 cm^{-1} of 0.08.

In Figure 6, the FTIR difference spectra of the 1700 cm^{-1} region, [spectrum time *t*] − [spectrum time zero], are shown as a function of time. The resulting spectra gives rise to a negative peak, corresponding to the biotin-bound CO_2^-, which increases in intensity over time. The FTIR difference spectra also have peaks in the positive direction, which increase in intensity with decarboxylation. In the present experiments, however, the biotin peaks are obscured by amide I and amide II features that derive from slow NH to ND exchange of the β-sandwich of the protein. The 1700 cm^{-1} region of the FTIR difference spectra [time t_x − time t_0] shows an unresolved doublet at 1695 and 1699 cm^{-1} that increases in negative intensity as decarboxylation occurs (Figure 6). We recently published a vibrational and theoretical analysis of carboxybiotin, free in solution, where the carboxylation was carried out by chemical means (*2*). A moderately intense single IR feature occurs at 1701 cm^{-1} due to the carboxylate asymmetric stretch coupled to the ureido carbonyl stretch. Thus, now we assign the doublet centered at 1698 cm^{-1} in Figure 6 to the asymmetric stretch of the CO_2^- group bound to biotin on the 1.3S protein. The rate of decarboxylation was measured based on changes in intensity of the 1698 cm^{-1} vibration (data not shown). The rate of the spontaneous decarboxylation process is 2.8×10^{-3} min^{-1}, giving a half-life of 247.5 min. The rate is two times faster than that calculated from the NMR studies (1.4×10^{-3} min^{-1}). This difference is ascribed to the different temperatures used for the two sets of experiments (22 °C for the FTIR, 5 °C for the NMR), and to the fact that the FTIR experiments were carried out in the absence of buffer whereas 100 mM phosphate was used for the NMR samples.

There are several interesting facets to the FTIR observations. The presence of two, albeit close, CO_2^- peaks in the asymmetric stretch region suggests that the group is in two slightly different environments. Both peaks are at slightly lower wavenumbers as compared to free carboxybiotin in D_2O (*2*). Thus, we have evidence supporting the NMR data that an interaction is occurring between the carboxylate group on biotin of the 1.3S protein and the remainder of the protein. This is where the time scale advantage of vibrational spectroscopy comes into play. Since the time scale of vibrational spectroscopy is subpicoseconds, the FTIR data provides an instantaneous "snapshot" and complications of exchange broadening, such as occur in NMR, are absent. Thus, we detect two populations, probably chemically similar, with slightly different environments about the CO_2^- group, where there are interactions between carboxybiotin and the protein. There is no evidence for a major population where the carboxybiotin is protruding away from the protein in an essentially aqueous environment, where it would give rise to the CO_2^- asymmetric stretch near 1701 cm^{-1}.

DISCUSSION

The N-terminus of the 1.3S subunit in solution, previously thought to be in a random coil/unordered conformation at neutral pH, in fact undergoes exchange between two or more ordered conformations. Thus, prior to carboxylation there is a well-structured C-terminus and a N-terminus that is exchanging between two or more conformational states resulting in loss of NMR signals from the N-terminus. Carboxylation of the biotin cofactors triggers structural changes in the protein, and these changes are detected by the appearance of new resonances, primarily due to asparagine side chains in the N-terminus, and by the changes in chemical shifts of resonances in both the 1D and 2D NMR spectra. The carboxylation of the biotin cofactor is responsible for the observed differences because the spectroscopic changes are fully reversible upon spontaneous decarboxylation. Once the carboxylate group leaves the biotin cofactor, the new resonances disappear and signatures due to non-carboxylated 1.3S reappear. In general, as decarboxylation occurs there is a tendency for many resonances throughout the 1D spectrum to intensify and become better resolved (data not shown). This is further evidence that carboxylation causes the 1.3S protein to change its static and/or dynamic conformational properties. These findings suggest that when the biotin acquires the negatively charged CO_2^- group it does interact with the N-terminus in some way and perturbs the conformational space that existed prior to carboxylation. The negative charge of the carboxylate group suggests that part of this perturbation may be electrostatic in nature. It is easy to see how interactions of the biotin-CO_2^- group with Lys-2 and Lys-4 would change the conformational properties of Asn-8 and, similarly, how the contact of biotin-CO_2^- with Lys-20 could perturb Asn-24. For the 1.3S protein, the shift in the asymmetric stretch of the CO_2^- group of carboxybiotin compared to free carboxybiotin (FTIR data) is further

indication that the carboxylate is interacting with the protein. The measured rate for the decarboxylation of the carboxylated-1.3S subunit, 2.8×10^{-3} min^{-1}, is lower than that measured rate for free carboxy-biotin (*21*) which has a decarboxylation rate at pH 6.4 of 4.6×10^{-3} min^{-1} at 25 °C. This is additional evidence that for the $1.3S-CO_2^-$ complex carboxybiotin−protein interactions are perturbing the environment about the carboxybiotin.

In conclusion, the observed sensitivity of the N-terminus to changes in pH or carboxylation of biotin may not reflect directly the behavior of the 1.3S subunit in the intact TC, since at least part of the N-terminus is anchored to the 12S and 5S subunits (*8*). However, the picture emerging from the present studies is of the 1.3S protein being in a highly sensitive "poised" conformational space that undergoes response to carboxylation. In this sense, similar effects may help bring about translocation of the 1.3S biotinylated β-sheet head during the catalytic cycle of the intact TC.

REFERENCES

1. Wood, H. G. (1979) *CRC Critical Reviews in Biochemistry*, 143−160.
2. Clarkson, J., and Carey, P. R. (1999) *J. Phys. Chem. A 103*, 2851−2856.
3. Reddy, D. V., Shenoy, B. C., Carey, P. R., and Sonnichsen, F. D. (1997) *Biochemistry 36*, 14676−14682.
4. Reddy, D. V., Rothemund, S., Shenoy, B. C., Carey, P. R., and Sonnichsen, F. D. (1999) *Protein Sci. 7*, 2156−2163.
5. Reddy, D. V., Shenoy, B. C., Carey, P. R., and Sonnichsen, F. D. (2000) *Biochemistry 39*, 2509−2516.
6. Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Haase, F. C., and Wood, H. G. (1988) *J. Biol. Chem. 263*, 6461−6464.
7. Athappilly, F. K., and Hendrickson, W. A. (1995) *Structure 3*, 1407−1419.
8. Ahmad, F., Jacobson, B., Chuang, M., Brattin, W., and Wood, H. G. (1975) *Biochemistry 14*, 1606−1611.
9. Stallings, W., Monti, C. T., Lane, M. D., and DeTitta, G. T. (1980) *Proc. Natl. Acad. Sci. 77*, 1260−1264.
10. Knowles, J. R. (1989) *Annu. Rev. Biochem. 58*, 195−221.
11. DeTitta, G. T., Edmonds, J. W., Stallings, W., and Donohue, J. (1976) *J. Am. Chem. Soc. 98*, 1920−1926.
12. Thatcher, G. R. J., Poirier, R., and Kluger, R. (1986) *J. Am. Chem. Soc. 108*, 2699−2704.
13. Shenoy, B. C., Magner, W. J., Kumar, G. K., Phillips, N. F. B., Haase, F. C., and Samols, D. (1993) *Protein Expression Purif. 4*, 85−94.
14. Thornton, C. G., Kumar, G. K., Haase, F. C., Phillips, N. F. B., Woo, S. B., Park, V. M., Magner, W. J., Shenoy, B. C., Wood, H. G., and Samols, D. (1993) *J. Bacteriol. 175*, 5301−5308.
15. Wood, H. G., Jacobson, B., Gerwin, B. I., and Northrop, D. B. (1969) in *Methods of Enzymology* (Lowenstein, J. M., Ed.) pp 215−230, Academic Press.
16. Piotto, M., Saudek, V., and Sklenar, V. (1992) *J. Biomol. NMR 2*, 661−665.
17. Kay, H. H., Hawkins, S. R., Gordon, J. D., Wang, Y., Ribeiro, A. A., and Spicer, L. D. (1992) *Am. J. Obstet. Gynecol. 167*, 548−553.
18. Maloy, W. L., Bowien, B. U., Zwolisnki, G. K., Kumar, G. K., Wood, H. G., Ericsson, L. H., and Walsh, K. A. (1979) *J. Biol. Chem. 254*, 11615−11622.
19. Murtif, V. L., Bahler, C. R., and Samols, D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5617−5621.
20. Ludlam, C. F., Arkin, I. T., Liu, l. X. M., Rothman, M. S., Rath, P., Aimoto, S., Smith, S. O., Engelman, D. M., and Rothschild, K. J. (1996) *Biophys. J. 70*, 1728−36.
21. Tipton, P. A., and Cleland, W. W. (1988) *J. Am. Chem. Soc. 110*, 5866−5869.

BI0116442