Productive Interactions between the Two Domains of Pig Heart CoA Transferase during Folding and Assembly†

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ABSTRACT: The enzyme CoA transferase from porcine heart (EC 2.8.3.5) is a homodimer; each subunit consists of two domains linked by a hydrophilic "hinge" region. We have prepared separate DNA segments encoding each of these domains. Incorporation of these two DNA segments within an operon or within two separate transcription units does not preclude the synthesis and assembly of CoA transferase in *Escherichia coli*. When the two domain fragments are produced and purified individually from separate cultures and subsequently mixed, enzyme activity accumulates to near wild-type levels only after a lengthy incubation. Each domain is more susceptible to aggregation than wild-type CoA transferase. Circular dichroism shows that, prior to mixing, the domains possess a different secondary structural profile compared to their counterparts in the native enzyme. However, mixing and incubation of the domains produces a complex with far-UV CD, fluorescence, and ultracentrifugation properties similar to those of wild-type CoA transferase. Finally, we show that the intact hydrophilic peptide which links the two domains is essential for the recovery of activity observed upon refolding of the denatured enzyme *in vitro*. These results indicate that the folding and assembly of pig heart CoA transferase require a productive interaction between its two domains, involving a substantial conformational rearrangement.

As a class of enzymes, the coenzyme A (CoA) transferases catalyze the reversible transfer of a CoA moiety between donor and acceptor carboxyl functions. Collectively, these enzymes are ubiquitous in nature and display a variety of substrate specificities that satisfy numerous metabolic requirements. For example, the activation of carboxylic acids by the transfer of CoA is crucial for the oxidation of some carbon sources in prokaryotes (Sramek & Frerman, 1975; Yeh & Ornston, 1981; Buckel et al., 1981; Wiesenborn et al., 1989; Scherf & Buckel, 1991; Petersen et al., 1993; Fischer et al., 1993; Kowalchuk et al., 1994; Parke, 1995) and for the metabolism of ketone bodies in mammalian tissues such as brain, heart, and kidney (White & Jencks, 1976; Sharp & Edwards, 1978; Russel & Patel, 1982). The ketone body acetoacetate is initially activated in the mitochondrial matrix by the transfer of a CoA moiety from succinyl-CoA, a reaction catalyzed by the homodimeric enzyme CoA transferase [succinyl-CoA:3-ketoacid coenzyme A transferase, EC 2.8.3.5.]. The subunit molecular weight of the mature protein is 52 197, as deduced from a cDNA encoding the cytoplasmic precursor of this enzyme (Lin & Bridger, 1992). Furthermore, a hydrophilicity plot for the CoA transferase monomer clearly suggests a structure in which two interacting domains are connected by a sequence of hydrophilic amino acid residues referred to as a "hinge" or "linker" segment (Lin & Bridger, 1992). This hinge is highly susceptible to proteolysis when the enzyme is purified in the absence of proteinase inhibitors, and cleavage in this region generates amino-terminal (N-domain) and carboxyl-terminal (C-domain) fragments with molecular weights of 2.8×10^4 and 2.4×10^4, respectively (Lin & Bridger, 1992). The "nicked" form of CoA transferase retains full catalytic activity (Lin & Bridger, 1992; Rochet & Bridger, 1994), demonstrating that the enzyme's catalytic function is not compromised by the lack of an intact hydrophilic linker segment in the folded protein.

In contrast to the mammalian enzymes, the CoA transferases from *Pseudomonas putida*, *Agrobacterium tumefaciens*, *Clostridium acetobutylicum*, and *Acinetobacter calcoaceticus* consist of two distinct polypeptides (designated α and β) organized as $\alpha_2\beta_2$ tetramers (Parales & Harwood, 1992; Peterson et al., 1993; Fischer et al., 1993; Kowalchuk et al., 1994; Parke, 1995). The genes encoding the α- and β-subunits of the prokaryotic enzyme are contained within one operon. Alignment of this operon with the cDNA encoding pig heart CoA transferase reveals substantial similarity in the resulting amino acid sequence between the prokaryotic α- and β-polypeptides and the N- and C-domains of the mammalian monomer, respectively (Parales & Harwood, 1992). A gap in the coding sequence of the prokaryotic operon exists in a region that corresponds to the hinge segment of the pig heart enzyme (Parales & Harwood, 1992). These observations suggest that the cDNA encoding pig heart CoA transferase may be the product of a fusion between two ancestral genes (Parales & Harwood, 1992). Given that

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[1] Abbreviations: CoA, coenzyme A; PCR, polymerase chain reaction; MCS, multiple cloning site; IPTG, isopropylthiogalactoside; 2ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; far-UV CD, far-ultraviolet circular dichroism; BS^3, bis(sulfosuccinimidyl)-suberate; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); GuHCl, guanidine hydrochloride; DTT, dithiothreitol; E_a, activation energy.

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an intact hinge connecting the N- and C-domains of the mammalian enzyme is not necessary to maintain a conformation with full catalytic activity, we hypothesized that this linker may instead facilitate an essential interaction between the two domains of CoA transferase during the enzyme's folding and assembly, prior to the achievement of its active configuration.

In order to test this hypothesis, we have carried out a number of studies to determine whether or not the N- and C-domains of pig heart CoA transferase fold independently of each other under different conditions. Initially, the production of the wild-type, recombinant enzyme in *Escherichia coli* was compared to that of a hinge-mutant form in which the N- and C-domains are assembled as separate polypeptides. Subsequently, each domain fragment was characterized according to its tendency to aggregate and its far-UV CD, fluorescence, and ultracentrifugation properties. These properties were also monitored, in parallel with the appearance of enzyme activity, in a mixture of the two domains incubated at various temperatures. Finally, wild-type CoA transferase and the hinge-mutant were compared in terms of their ability to refold upon dilution from denaturant. The results of these experiments suggest that a productive interaction between the N- and C-domains involves a conformational rearrangement, necessary for the proper folding and assembly of pig heart CoA transferase. We also show that the hydrophilic linker, while not required for the assembly of the enzyme *in vivo*, may play a key role in facilitating the interaction between the N- and C-domains during refolding *in vitro*.

EXPERIMENTAL PROCEDURES

Materials. The restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Inc. or as Gibco BRL products from Life Technologies, with no obvious differences. Acrylamide and *Taq* DNA polymerase were also obtained as Gibco BRL products from Life Technologies. The deoxynucleotide triphosphates, Sephadex G-50, and Sephacryl S-200 were purchased from Pharmacia Biotech. The Sequenase was supplied by the United States Biochemical Corporation. The T4 DNA polymerase and the Lumi-Phos 530 were obtained from Boehringer Mannheim, Inc. The plasmid pT7-7 was a gift from Dr. Stanley Tabor, while pMS119EH and pKIXX were generously provided by Dr. Raymond Turner. The column matrices DEAE Sephacel, AffiGel-Blue, and hydroxyapatite were purchased from Bio-Rad Laboratories, Ltd. Purified protein preparations were concentrated using Amicon brand centricon concentrators (MWCO = 1.0×10^4). Radioactively labeled ^{35}S-methionine (typically 5−10 mCi/mL) was supplied by ICN Biomedicals, Inc. as ^{35}S-TransMet or by Amersham Ltd. The substrates acetoacetate and succinyl-CoA were purchased from Sigma Chemical Company.

Subcloning and Mutagenesis. A flow diagram of these procedures is presented in Figure 1. Firstly, a 1.5 kb segment of the λT6 cDNA clone (Lin & Bridger, 1992) encoding the mature form of CoA transferase was amplified by the polymerase chain reaction (PCR), using the primers NDE and HIND. An expression plasmid for the production of wild-type enzyme (pT7-WT) was then created by subcloning the amplified DNA as an *Nde*I−*Hin*dIII fragment into the multiple cloning site (MCS) of pT7-7. M13-WT was then prepared for subsequent mutagenesis experiments by transferring the 1.5 kb *Xba*I−*Hin*dIII segment of pT7-WT into the MCS of M13-MP19. Secondly, an M13 vector containing a gene to encode the hinge-mutant form of CoA transferase (M13-Hng) was generated by site-directed mutagenesis according to previously described methods (Kunkel, 1985), using M13-WT DNA as the single-stranded template and INT-1 as the mutagenic primer. This primer included a stop codon and a reinitiation site in the region of the DNA encoding the hydrophilic linker segment. Due to the design of this primer, the hinge-mutant form of CoA transferase lacked residues 249−254, which are normally present in the linker segment of the wild-type enzyme. (Thus, the predicted molecular weight of the N-domain of the hinge-mutant was 2.7×10^4.) The 0.9 kb *Bam*HI−*Nco*I segment cleaved from M13-Hng was subcloned into pT7-WT, yielding pT7-Hng. Thirdly, an expression vector for the production of the C-domain of CoA transferase was prepared by digestion of pT7-Hng with *Nde*I and subsequent religation, yielding the vector pT7-C. (The predicted molecular weight of the C-domain was 2.4×10^4.) Finally, a DNA insert encoding the N-domain of CoA transferase was amplified from pT7-Hng by the PCR, using the primers NDE and INT-2. The latter was designed such that a stop codon associated with a *Hin*dIII restriction site was incorporated in the region of the DNA insert encoding the hydrophilic linker segment. An expression plasmid for the production of the N-domain was then prepared by subcloning the amplified DNA as a 0.16 kb *Bst*XI−*Hin*dIII fragment into pT7-WT, yielding pT7-7-N.

A parallel series of expression plasmids was generated by transferring an *Xba*I−*Hin*dIII segment from each pT7-7-derived vector into the MCS of pMS-119EH or its kanamycin-resistant variant, pMK119EH. The latter was constructed by replacing the 0.7 kb *Dra*I fragment of pMS119EH (which included most of the gene encoding β-lactamase) with the 1.2 kb *Sma*I fragment of pKIXX (which encoded a marker for resistance to kanamycin).

The vector pMS-C/N, in which the genes encoding the N- and C-domains of CoA transferase are incorporated within two separate transcription units, was derived from pMS-N and pMK-C. Specifically, the 1.4 kb *Mlu*I−*Hin*dIII insert of pMK-C was attached in two steps to the 4.4 kb fragment cleaved from pMS-N with *Mlu*I and *Pfl*MI: (1) ligation of the complementary *Mlu*I sites on the two fragments and (2) repair of the *Hind*III site on the small fragment and the *Pfl*MI site on the large fragment with T4 DNA polymerase, followed by ligation of the resulting blunt ends.

The absence of possible mutations due to misincorporation by *Taq* DNA polymerase or Sequenase during the *in vitro* DNA synthesis reactions was confirmed by automated sequence determinations using previously described methods (Sanger et al., 1977) and an Applied Biosystems (ABI) 373A DNA sequencer.

Electrocompetent BL21(DE3) cells (*hsdS gal* [λcI*ts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene*1*]) were electroporated with vectors of the pT7-7 series, yielding the new strains BL-WT, BL-Hng, BL-N, and BL-C. Similarly, electrocompetent HB101 cells (*supE*44 *hsdS*20[$r_B^-m_B^-$] *rec*A13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1) were electroporated with vectors of the pMS119-EH series, yielding the new strains HB-WT, HB-Hng, HB-N, HB-C, HB-N+C, and HB-C/N.

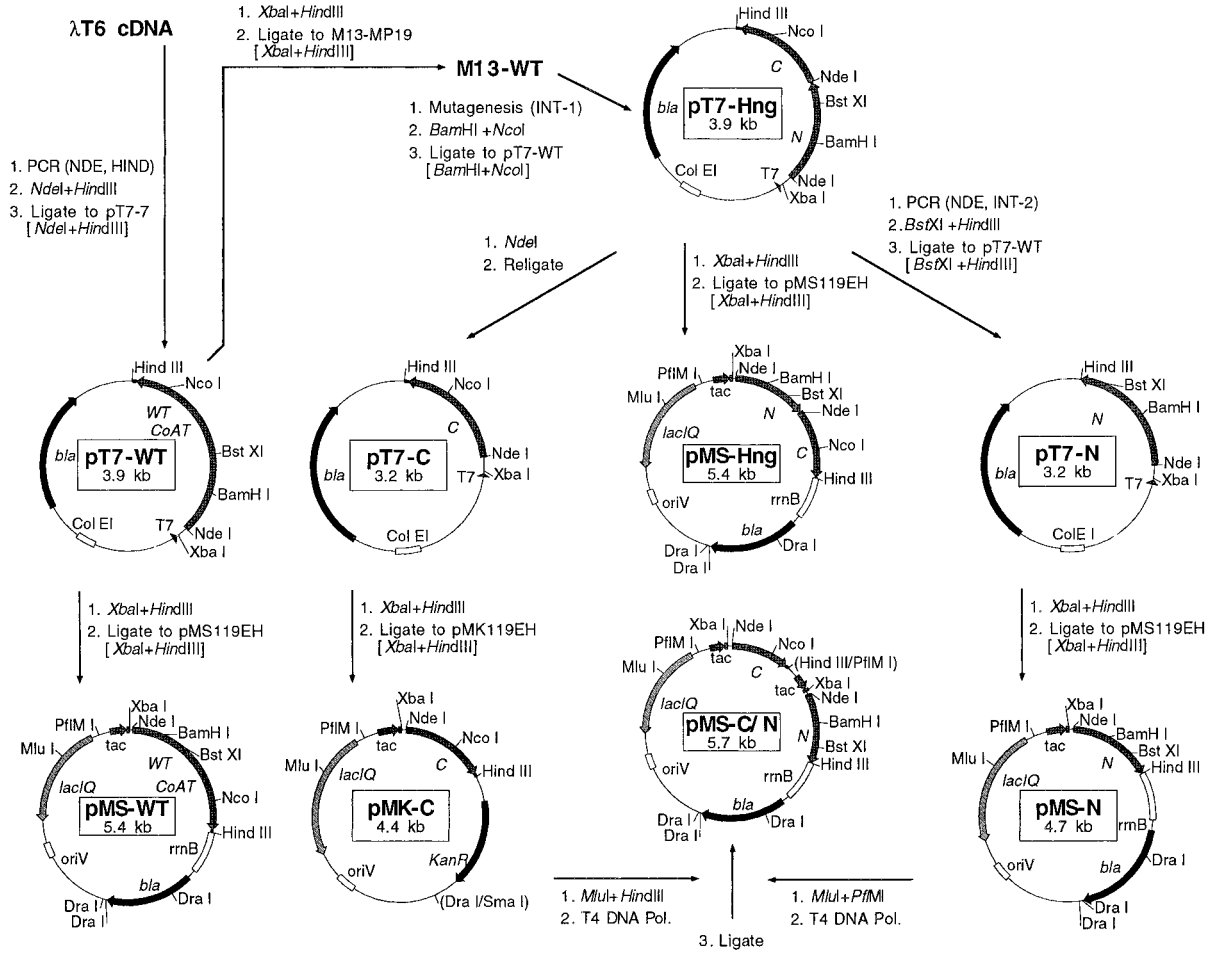

FIGURE 1: Construction of expression vectors encoding CoA transferase polypeptides. The primers used for PCR were NDE (5′-**CGCGGCATATGACCAAATTTTATACGGATGC**-3′), HIND (5′-**GCACAAAGCTTGGGACAAATATCTGTTCC**-3′), and INT-2 (5′-**CAGACATATAAGCTTACTCCTTCCGGACTGATAA**-3′). The vector pT7-Hng was created by site-directed mutagenesis using single-stranded M13-WT as the template and INT-1 (5′-**TTATCAGTCCGGAAGGAGTAAAATCATATGTCTGGTAAACTTGGA**-3′) as the mutagenic primer (Kunkel, 1985). ColE1 and oriV designate origins of DNA replication, while T7 and *tac* refer to promoter sequences. *KanR* and *bla* designate genes conferring resistance to kanamycin and ampicillin, respectively. Other encoded proteins are identified as follows: *lacI*^{Q}, lac repressor; *WT CoAT*, wild-type CoA transferase; *Hng*, hinge-mutant; *N*, N-domain; *C*, C-domain. (*Hin*dIII/*Pfl*MI) and (*Dra*I/*Sma*I) refer to restriction sites destroyed as a result of blunt-end ligation. "T4 DNA Pol." is an abbreviation for T4 DNA polymerase.

Gene Expression. Cells of *E. coli* BL21(DE3) or HB101 were grown at 37 °C in Luria Broth (LB) supplemented with ampicillin (final concentration, 100 μg/mL). Gene expression was induced in cultures at the mid-log phase by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 100 μM for BL21 cells or 1 mM for HB101 cells. HB101 cells containing two plasmids, one derived from pMS119 and the second derived from pMK119, were grown in the presence of 100 μg/mL ampicillin and 40 μg/mL kanamycin. Following growth at 25 °C for 8−12 h, the cells were collected by centrifugation (10 min, 4×10^3 g), and the cell pellets were resuspended in buffer S (0.05 M K[phosphate], 0.5 mM EDTA, 10 mM 2-mercaptoethanol [2ME], pH 7.4) and frozen at −70 °C. After thawing, the suspensions were sonicated at 4 °C, and the cell debris was separated from the cell lysate by centrifugation (10 min, 4×10^3 g). Generally, the protein concentration of the cell lysate prepared in this manner was 0.5−1 mg/mL. The relative amounts of CoA transferase protein present in the pellet and soluble fractions were determined by immunoblot analysis using the Lumi-Phos chemiluminescent detection system. The results from these blots were quantified by densitometric scanning using a Joyce Loebl Chromoscan 3.

Protein Purification. All protein purification procedures were carried out at 4 °C. The fraction of the cell lysate which was soluble at 18% (w/v) $(NH_4)_2SO_4$ but insoluble at 50% (w/v) $(NH_4)_2SO_4$ was dissolved in buffer A (0.01 M K[phosphate], 0.1 mM EDTA, 10 mM 2ME, pH 7.4) or buffer D (0.005 M Tris-HCl, 0.1 mM EDTA, 10 mM 2ME, pH 8.0). This protein solution (3−5 mg/mL) was desalted by gel exclusion chromatography on a Sephadex G-50 column prior to further purification.

Wild-type CoA transferase and the hinge-mutant were purified as previously described (Lin & Bridger, 1992) with successive fractionations on columns of DEAE-Sephacel, Sephacryl S-200, and AffiGel Blue. Optimally, a 2 L culture of BL-WT or BL-Hng yielded 10 mg of pure CoA transferase or 30 mg of pure hinge-mutant, respectively.

The N- and C-domains of CoA transferase were purified by modifications of the chromatographic procedure referenced above for the wild-type enzyme or hinge-mutant. The N-domain was eluted from (1) DEAE-Sephacel, using a gradient of 0 to 0.5 M KCl in buffer D; (2) Sephacryl S-200, using buffer A; and (3) hydroxyapatite, using a gradient of 0.01 to 0.4 M K[phosphate] in buffer A. The C-domain was eluted from (1) AffiGel Blue, using a gradient of 0 to 1.5 M

KCl in buffer A; (2) Sephacryl S-200, using buffer A; and (3) hydroxyapatite, using a gradient of 0.01 to 0.4 M K[phosphate] in buffer A. After each chromatographic step, fractions containing the N- or C-domain in purest form were identified by SDS−PAGE analysis and subsequently pooled. Optimal yields were 80 mg of pure N-domain or 20 mg of pure C-domain isolated from 2 L cultures of HB-N or HB-C, respectively.

Pure protein solutions were concentrated by centrifugation (2×10^3 g) using Centricon concentrators (MWCO = 1.0×10^4). The homogeneity of all protein preparations was confirmed by SDS−PAGE analysis. The concentrations of the purified proteins were determined spectrophotometrically using the following extinction coefficients (ϵ_{278}), in units of (inverse milligrams per milliliter): 0.65 for the wild-type enzyme, 0.66 for the hinge-mutant, 0.63 for the N-domain, and 0.69 for the C-domain. These values were calculated based on the molecular absorbances of the aromatic amino acids, as previously tabulated (Mihalyi, 1968), and were confirmed using the fringe-count method (Babul & Stellwagen, 1969).

Measurement of Enzyme Activity. CoA transferase activity was determined spectrophotometrically as described previously (Stern et al., 1956; Rochet & Bridger, 1994). In the present study, the assay solutions consisted of 50 mM Tris-HCl, 15 mM $MgCl_2$, 60−300 μM succinyl-CoA, and 13−67 mM acetoacetate, at pH 9.1. All assays were carried out at 22 °C.

Preparation of N- and C-Domain Mixtures. Appropriate amounts of the two domains were initially mixed at 4 °C in preparation for folding studies at higher temperatures. Once the incubation temperature was raised, aliquots were removed at specific times, cooled immediately, and stored at 4 °C. After several days, all aliquots were assayed for CoA transferase activity under standard conditions. Results from control experiments indicated that no increase or decrease in enzyme activity occurred over background levels in mixtures of the domains stored at 4 °C for periods of at least 6 weeks.

A sample of N_2C_2 complex suitable for detailed biophysical analyses (including CD, fluorescence, ultracentrifugation, and light scattering studies) was generated by incubating an equimolar mixture of the N- and C-domains at 22 °C for 4 h. This sample was subsequently purified by gel filtration through a Sephacryl S-200 column.

Amino Acid Analysis, Edman Degradation Sequencing, and Mass Spectrometry. Amino acid analysis was carried out using standard procedures on a ninhydrin-based Beckmann 6300 instrument. Protein sequence analysis was performed using a 473A pulsed liquid/gas phase microsequencer from Applied Biosystems Incorporated (Foster City, CA). Standard cycles (ABI version 2.00) and Edman chemistry were used. Mass analyses were performed on a Fisons VG Quattro triple quadrupole mass spectrometer (Manchester, U.K.) fitted with an electrospray ionization source operating in positive ion mode. Protein samples (10 μL, 50 pmol/μL in 0.2M K[phosphate], 0.1 mM EDTA, and 10 mM 2ME, pH 7.4) were injected into the electrospray source at a rate of 10 μL/min via a carrier solution of water: acetonitrile (1:1, v/v) containing 0.05% (v/v) TFA. The quadrupoles were scanned at 10 s/scan for mass over charge ratios ranging from 600 to 1400. Data were acquired in the MCA mode, and 10−15 scans were typically summed to produce the final spectrum.

Pulse-Chase Experiments. The stability of CoA transferase proteins in *E. coli* BL21(DE3) was assessed at 37 °C by pulse-chase experiments using standard procedures for exclusive labeling with ^{35}S-methionine (Tabor & Richardson, 1985). Aliquots of cells (200 μL) were removed from the cultures at different times following chase and lysed with Triton X-100 according to published methods (Horwich et al., 1993). Subsequently, a portion of each cell lysate was fractionated by SDS−PAGE, and the amount of radioactively labeled protein quantified by phosphorimager analysis.

Circular Dichroism and Fluorescence Studies. Far-UV circular dichroism (CD) measurements were carried out using a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced to an Epson Equity 386/25 computer and controlled by Jasco software. The thermostated cell holder was maintained at 25 °C with a Lauda RMS circulating water bath (Lauda, Westbury, NY). The instrument was routinely calibrated with ammonium *d*-(+)-10-camphorsulfonate at 290.5 nm and 192 nm and with *d*-(−)-pantoyllactone at 219 nm. Each protein sample (0.5−1 mg/mL in 0.2 M K[phosphate], 0.1 mM EDTA, 10 mM 2ME, pH 7.4) was scanned 10 times, and noise reduction was applied to remove the high frequencies before calculating molar ellipticities. The voltage of the photomultiplier tube was kept below 500 V to prevent distortion of the CD spectrum. A calibrated cell of 0.02 cm path length was used for all measurements. The following mean residue weights were assumed: wild-type enzyme, 108.687; hinge-mutant, 108.526; N-domain, 109.171; C-domain, 107.900; N + C complex (1:1), 108.563. Fluorescence analyses were performed using a Perkin-Elmer MPF-44B spectrofluorometer linked to a differential corrected spectra unit (DSCU2). Temperature control was achieved by means of a thermostated cell holder and a Lauda RMS water bath. Baseline-corrected excitation and emission spectra were recorded using a band width of 8 nm. Protein samples (0.07 mg/mL in 0.2 M K[phosphate], 0.1 mM EDTA, 10 mM 2ME, pH 7.4) were excited at 295 nm in order to minimize energy-transfer effects due to proximal tyrosines. Absorbance measurements were performed on a Perkin−Elmer Lambda 5 spectrophotometer.

Cross-Linking Studies. Purified proteins (diluted to 1 mg/mL in 0.15 M NaCl, 1 mM DTT) were incubated for 4−5 h at 4 °C with bis(sulfosuccinimidyl)suberate (BS^3) or 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) at final cross-linker concentrations of 1−2 mM. The reactions were quenched by the addition of Tris-HCl (7.4) to a final concentration of 50 mM. Cross-linked samples were then analyzed by SDS−PAGE under both reducing and nonreducing conditions (DTSSP-cross-linked proteins) or under reducing conditions only (BS^3-cross-linked proteins) and visualized by staining with Coomassie Brilliant Blue.

Sedimentation Equilibrium. Molecular weight determinations were made using the conventional sedimentation equilibrium technique (Chervenka, 1970). All solutions of proteins (1−2 mg/mL in 50 mM K[phosphate], 150 mM KCl, 1 mM EDTA, 10 mM 2ME, pH 7.4) were analyzed at 20 °C except for the C-domain, which was analyzed at 10 °C to avoid complications due to aggregation. Runs were carried out for 48 h using a Beckman Model E Analytical Ultracentrifuge equipped with Rayleigh interference optics. The initial concentration of protein was determined by the

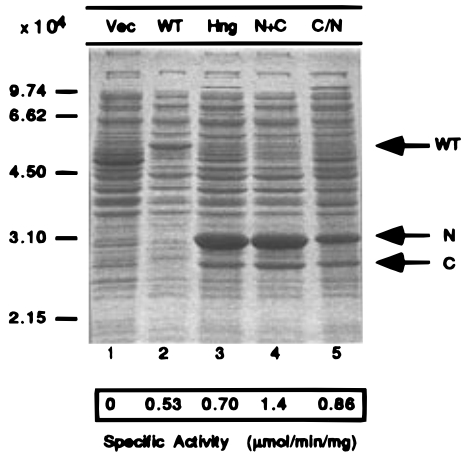

FIGURE 2: Production of soluble CoA transferase polypeptides in *E. coli*. Lysates from cells grown for 8 h at 25 °C in the presence of 1 mM IPTG were analyzed by SDS−PAGE with Coomassie Blue staining (top) and enzyme assay (bottom). Lane 1, HB-pMS119EH vector control; lane 2, HB-WT; lane 3, HB-Hng; lane 4, HB-N+C; lane 5, HB-C/N. Each lane was loaded with 30 μg of total cell lysate protein. Bands representing the 5.2×10^4 wild-type subunit (WT), 2.7×10^4 N-domain (N), and 2.4×10^4 C-domain (C) are indicated by appropriately labeled arrows. Enzyme activities were measured with an assay solution of 50 mM Tris-HCl, 15 mM $MgCl_2$, 300 μM succinyl-CoA, and 67 mM acetoacetate, at pH 9.1.

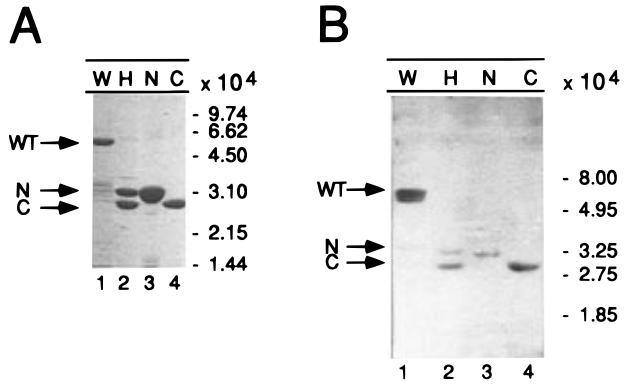

FIGURE 3: Purification of CoA transferase polypeptides. Proteins (3−5 μg) were analyzed by SDS−PAGE with Coomassie Blue staining in (A) or Western blotting in (B). Lane 1, wild-type CoA transferase (W); lane 2, hinge-mutant (H); lane 3, N-domain (N); lane 4, C-domain (C). Bands representing the 5.2×10^4 wild-type subunit (WT), 2.7×10^4 N-domain (N), and 2.4×10^4 C-domain (C) are indicated by appropriately labeled arrows.

fringe count method (Babul & Stellwagen, 1969), assuming 4.1 fringes/mg of protein. Apparent weight-average molecular molecular weights were calculated by fitting ln y vs r^2 data to a second-degree polynomial equation using least-squares techniques. The partial specific volume for each sample was calculated from the corresponding amino acid composition (Cohn & Edsall, 1943).

Light Scattering Procedures. Molecular weight determinations using light scattering techniques were performed on a Dawn F multiangle laser light scattering photometer (Wyatt Technology Corporation, Santa Barbara, CA), according to a previously described method (Wyatt, 1993).

Refolding Studies in vitro. Wild-type CoA transferase (final concentration of the NC monomer, 10 μM) and the hinge-mutant (final concentration of each domain, 10 μM) were denatured in unfolding buffer (6.0 M guanidine hydrochloride [GuHCl], 0.09 M Tris-HCl, 0.045 M KCl, 0.09 mM EDTA, and 1 mM DTT, pH 8.0) at 22 °C for a minimum of 5 h. The solutions of the unfolded proteins were then diluted 1:50 in refolding buffer (0.09 M Tris-HCl, 0.045 M KCl, 0.09 mM EDTA, and 1 mM DTT, pH 8.0). Alternatively, the solutions of the unfolded enzymes were placed on a semipermeable membrane (Millipore VM, pore size 0.05 μm) and dialyzed against 600 mL of the same refolding buffer. In both cases, the samples were incubated for 90 min at 22 or 37 °C prior to being assayed for enzyme activity.

Data Analysis. Kinetic data (Figures 4, 5, and 6) were fit to single- or double-exponential curves using the program TableCurve2D (Jandel Scientific, San Rafael, CA). The data and best-fit curves were subsequently plotted using the program SigmaPlot (also from Jandel).

RESULTS

Synthesis of CoA Transferase Forms in E. coli. To generate the material necessary to examine the folding properties of pig heart CoA transferase *in vivo*, we first prepared the plasmids pT7-WT and pMS-WT, both of which encode the wild-type enzyme in its mature form (Figure 1). Subsequent transfection of *E. coli* HB101 with pMS-WT yielded the strain HB-WT. By measurements of enzyme activity and immunoreactive protein, we determined that maximal levels of soluble CoA transferase were produced in HB-WT cells grown in the presence of 1 mM IPTG for 8−12 h at 25 °C (data not shown). Lysates recovered from these cells typically yielded 0.5 unit (μmol/min) of enzyme activity/mg of total protein and were correspondingly enriched with a polypeptide whose apparent molecular weight (estimated by SDS−PAGE) was similar to the value of 5.2×10^4 predicted for mature pig heart CoA transferase (Figure 2, lane 2 versus lane 1).

If the hydrophilic segment linking the N- and C-domains of CoA transferase is necessary for the proper folding of the enzyme, then a mutant form of CoA transferase in which the N- and C-domains are physically separated (referred to as the hinge-mutant) should not be properly assembled *in vivo*. A gene encoding such a hinge-mutant was constructed by incorporation of a stop codon and a reinitiation site in the portion of the DNA corresponding to the hydrophilic linker (see Experimental Procedures and Figure 1). This mutant gene was subsequently expressed in the strain HB-Hng under the conditions described above. Two proteins with apparent molecular weights similar to those predicted for the N- and C-domains (2.7×10^4 and 2.4×10^4, respectively) were evident in the lysates prepared from these cells and examined by SDS−PAGE (Figure 2, lane 3). The specific enzyme activity measured in these lysates was comparable to that observed in lysates recovered from the strain HB-WT (Figure 2, lane 3 versus lane 2). Moreover, the specific activity of the hinge-mutant following purification was equivalent to that of purified wild-type CoA transferase. These results indicate that an intact linker region is not essential for the formation of active CoA transferase produced in *E. coli*.

Next, we considered the possibility that the hinge-mutant is correctly folded and assembled only upon translation of a polycistronic mRNA, which might permit a crucial interaction between the two nascent polypeptide chains prior to or immediately following the completion of protein synthesis.

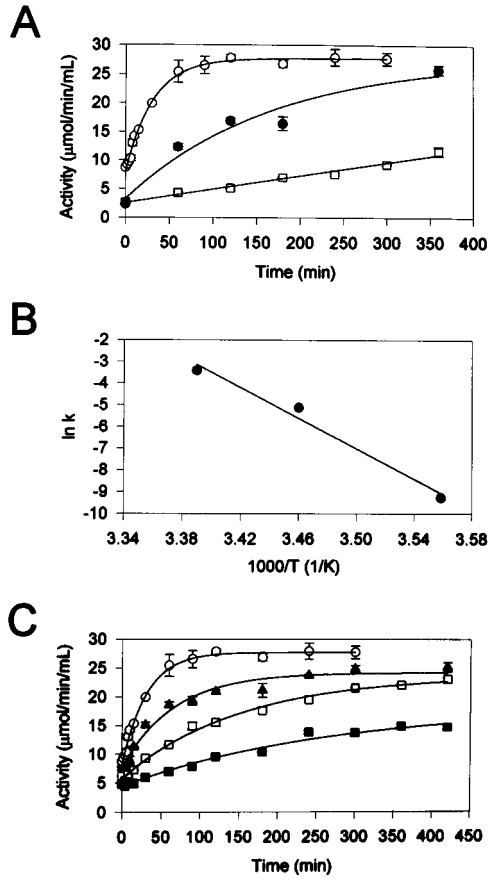

FIGURE 4: Kinetics of the formation of active CoA transferase in equimolar mixtures of the N- and C-domains. (A) Appearance of enzyme activity in mixtures of the purified N- and C-domain polypeptides (100 μM each). The mixtures were incubated at 8 °C (□), 16 °C (●), and 22 °C (○). The apparent first-order rate constants are 8 °C, $k = 1.6 \times 10^{-6}$ s^{-1} ($r^2 = 0.98$); 16 °C, $k = 9.8 \times 10^{-5}$ s^{-1} ($r^2 = 0.95$); and 22 °C, $k = 5.4 \times 10^{-4}$ s^{-1} ($r^2 = 0.99$). (B) Arrhenius replot of the apparent first-order rate constants in panel A, from which an E_a of $(2.9 \pm 0.4) \times 10^2$ kJ/mol was calculated ($r^2 = 0.98$). (C) Concentration dependence of the rate of enzyme formation. Mixtures of the purified N- and C-domain polypeptides, each at 10 μM (■), 20 μM (□), 50 μM (▲), or 100 μM (○), were incubated at 22 °C. The apparent first-order rate constants are 10 μM, $k = 6.1 \times 10^{-5}$ s^{-1} ($r^2 = 0.98$); 20 μM, $k = 1.1 \times 10^{-4}$ s^{-1} ($r^2 = 0.99$); 50 μM, $k = 2.5 \times 10^{-4}$ s^{-1} ($r^2 = 0.98$); and 100 μM, $k = 5.4 \times 10^{-4}$ s^{-1} ($r^2 = 0.99$). All experiments were performed in duplicate, with an average relative error of ±3% in panels A and C. Enzyme activities were measured using an assay solution of 50 mM Tris-HCl, 15 mM $MgCl_2$, 60 μM succinyl-CoA, and 13 mM acetoacetate, at pH 9.1.

If this were true, then active enzyme would not be assembled from N- and C-domain proteins encoded within separate transcription units. This hypothesis was first addressed by generating two plasmids (pMS-N and pMK-C), each containing, respectively, the gene for the N- and C-domain of CoA transferase (see Experimental Procedures and Figure 1). These genes were subsequently coexpressed in a strain of *E. coli* (HB-N+C) which harbors both plasmids. Analysis of the lysates from these cells by SDS−PAGE revealed that the N- and C-domain polypeptides were present at levels similar to those observed in the soluble fraction prepared from HB-Hng cells (Figure 2, lane 4). The presence of these proteins in the HB-N+C cell lysates once again correlated with a substantial amount of CoA transferase activity. Finally, due to uncertainties concerning the relative copy-number of the pMS-N and pMK-C vectors in the strain HB-N+C, we designed the plasmid pMS-C/N to carry the genes

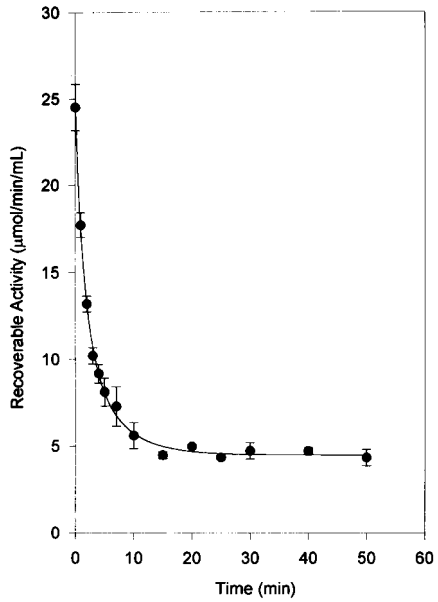

FIGURE 5: Aggregation of the N- and C-domains *in vitro*. Mixtures of the purified N- and C-domain polypeptides (100 μM each) were preincubated at 37 °C for the times shown. Subsequently, each sample was incubated further at 22 °C for 2 h prior to being assayed for enzyme activity. The data were fit to a double-exponential equation ($A = 4.49 + 12.2e^{-0.65T} + 7.87e^{-0.19T}$; $r^2 = 1.00$), where A = CoA transferase activity (micromoles per minute per milliliter) and T = time of preincubation at 37 °C (minutes). The experiment was performed in duplicate, with an average relative error of ± 7%. Enzyme activities were measured using an assay solution of 50 mM Tris-HCl, 15 mM $MgCl_2$, 60 μM succinyl-CoA, and 13 mM acetoacetate, at pH 9.1.

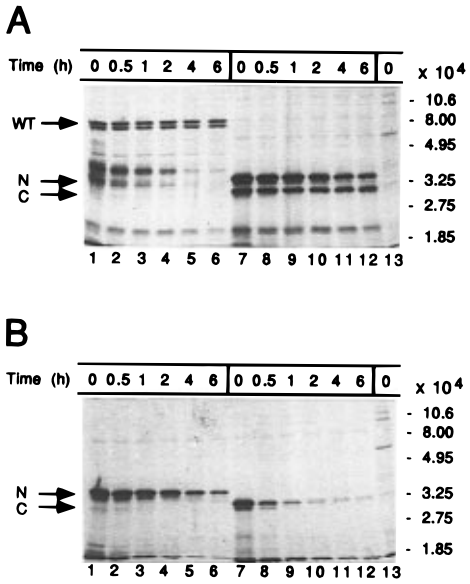

FIGURE 6: Aggregation of the N- and C-domains *in vivo*. (A) Cultures of BL-WT (lanes 1−6), BL-Hng (lanes 7−12), and BL-pT7-7 (lane 13) were pulsed at 37 °C with ^{35}S-methionine prior to chase for the indicated times. Cell lysates were subsequently analyzed by SDS−PAGE and autoradiography. Each lane was loaded with 3 μg of total cell lysate protein. Bands representing the 5.2×10^4 wild-type subunit (WT), 2.7×10^4 N-domain (N), and 2.4×10^4 C-domain (C) are indicated by appropriately labeled arrows. (B) Cultures of BL-N (lanes 1−6), BL-C (lanes 7−12), and BL-pT7-7 (lane 13) were treated and evaluated as in panel A. Bands representing the 2.7×10^4 N-domain (N) and 2.4×10^4 C-domain (C) are indicated by appropriately labeled arrows.

encoding the N- and C-domains downstream of two separate promoters (see Experimental Procedures and Figure 1).

Results similar to those described above were also observed in cell lysates recovered from the strain HB-C/N (Figure 2, lane 5). Thus, the N- and C-domains of the hinge-mutant of CoA transferase need not be synthesized from sequential genes within the same operon in order to assemble correctly and form active enzyme *in vivo*.

Mixtures of the Purified N- and C-Domains. Given our observation that the N- and C-domains encoded from separate transcription units are properly assembled in *E. coli*, we proposed that each domain may fold independently of the other, such that their final association consists of the docking of two polypeptides possessing their native folds. If this were true, then this docking event should be reproduced *in vitro* by mixing the two purified domains, leading to the rapid generation of full CoA transferase activity.

The individual N- and C-domains were isolated from the strains HB-N and HB-C (respectively), using methods previously described for the purification of wild-type CoA transferase. A visual demonstration of the final purity of these two proteins by SDS−PAGE is provided in Figure 3A. Furthermore, each domain was positively identified by (1) Western blot analysis using a polyclonal antiserum raised against CoA transferase (Figure 3B) and (2) N-terminal amino acid sequencing. Finally, molecular masses of 27 049 and 24 464 Da were determined for the N- and C-domains (respectively) by electrospray mass spectrometry. Each of these values corresponded very closely to the molecular weight predicted for the N- or C-domain polypeptide lacking its N-terminal methionine, thus confirming the absence of additional post-translational modifications.

We examined whether CoA transferase activity is generated by mixing equimolar amounts of the purified N- and C-domains. Initially, these domains were isolated in the absence of 2ME, given our observation that the wild-type enzyme is not adversely affected by the omission of this reductant. A mixture of N- and C-domains, which had been purified in this manner yielded no CoA transferase activity, even following a lengthy incubation at 22 °C. In contrast, a progressive, panels increase in enzyme activity with apparent first-order kinetics (Figure 4A,B) was observed upon combining N- and C-domains (final concentration of each, 100 μM) that had been isolated in the presence of 2ME. A maximal specific activity of about 85% that of wild-type CoA transferase accumulated after a 2 h incubation at 22 °C. Repeating this experiment at different temperatures enabled us to calculate a value of $(2.9 \pm 0.4) \times 10^2$ kJ/mol for the activation energy (E_a) associated with this process (Figure 4B). At 37 °C much of the protein became insoluble, and very little activity was recovered. Furthermore, no increase in activity was detected above background levels in mixtures incubated at 4 °C for extended periods.

Finally, the kinetics of assembly of the N- and C-domains to the active enzyme form were investigated. Activity measurements were performed on equimolar mixtures of the domains which had been diluted 1/2, 1/5, and 1/10 prior to their incubation for different lengths of time at 22 °C. Enzyme activity appeared with apparent first-order kinetics in all samples examined (Figure 4C). The fact that second-order kinetics were not observed suggests that the incubation required for the generation of active CoA transferase cannot be attributed to the irreversible association of the N- and C-domains already possessing a native fold. Instead, the observed rate of activity production may be limited by a structural rearrangement in the domains following their initial interaction. Preincubation of the individual domains at 22 °C did not alter the observed kinetics, indicating that each domain only achieves its native fold in the presence of the other.

Aggregation Properties of the N- and C-Domains. Non-native polypeptides generally display an increased tendency to aggregate relative to their native counterparts (Jaenicke, 1982, 1991; Kim & Baldwin, 1982; Zettlmeissl et al., 1984). Accordingly, we attempted to verify our initial conclusion that the purified N- and C-domains lack a native structure by comparing their solubility (both *in vitro* and *in vivo*) to that of the correctly folded hinge-mutant.

We examined the effect of temperature on the solubility of the two purified domains *in vitro*. As mentioned previously, the mixed domains aggregated at 37 °C, and only low levels of enzyme activity were observed. The kinetics of this aggregation were evaluated by temperature-shift experiments, in which an equimolar mixture of the N- and C-domains was preincubated at 37 °C for various times before transfer to 22 °C for an additional 2 h. The loss of recoverable enzyme activity with increasing time of preincubation at 37 °C was best described by a double-exponential equation and was essentially complete at 15 min (Figure 5). In contrast, the purified hinge-mutant retained 85% of its initial enzyme activity when incubated at 37 °C for 1 h and subsequently at 22 °C for 3 h (data not shown). When the two protein domains were heated individually, both were found to precipitate at lower temperatures than the purified hinge-mutant; the C-domain aggregated at a lower temperature than the N-domain. From these results, we conclude that the N- and C-domains are more thermally unstable *in vitro* as separate polypeptides than as components of the correctly folded hinge-mutant and that the C-domain is more susceptible to heat denaturation than the N-domain.

The disappearance of the two domains from the soluble fraction was evaluated *in vivo* by pulse-chase labeling experiments. Cultures of BL-WT, BL-Hng, BL-N, and BL-C, grown at 37 °C, were treated briefly with ^{35}S-methionine, prior to dilution of the label by the addition of excess unlabeled methionine. At different times during the chase period, cell lysates were prepared from aliquots of each culture. Subsequent analysis by SDS−PAGE and autoradiography showed that the exclusively labeled, individual N- and C-domains decayed more rapidly than wild-type CoA transferase or the C-domain of the hinge-mutant (Figure 6). Quantitation of these results by phosphorimaging indicated that, for each of the proteins examined, the rate of disappearance of the label was best described by a double-exponential equation, which precluded a simple calculation of the half-life for this process. Nevertheless, approximately 50% of the radioactivity associated with wild-type CoA transferase or the C-domain of the hinge-mutant was lost after 5 h, while label disappeared to a similar extent from the individual N- or C-domains within only 2 or 0.25 h, respectively. It should be noted that the decay of the N-domain (apparent with BL-Hng in Figure 6A) did not reflect the disappearance of the hinge-mutant, *per se*. Because the N-domain polypeptide has only one quarter of the methionine residues of the C-domain, it follows that the bands of similar intensity in Figure 6A (lanes 7 through 12) did not originate from similar amounts of the two proteins; rather, the N-domain was present in large excess over the

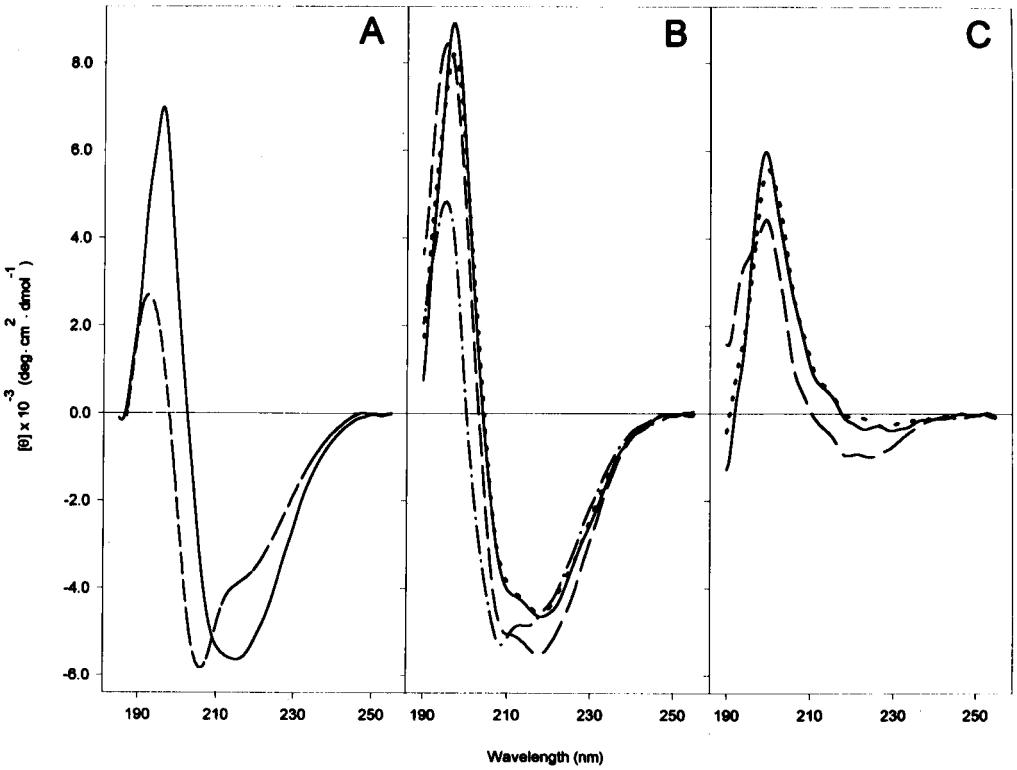

FIGURE 7: Analysis of the assembly of the N- and C-domains by far-UV CD. (A) Spectra representing the N-domain (—) and C-domain (---). (B) Spectra representing wild-type CoA transferase (---), the hinge-mutant (—), the complex of N- and C-domains formed at 22 °C (···), and the summed N- and C-domain curves (— · —). (C) Difference spectra derived by subtracting the summed N- and C-domain curves from the curves recorded for wild-type CoA transferase (---), the hinge-mutant (—), and the complex of N- and C-domains formed at 22 °C (···).

C-domain in BL-Hng. It was this excess of N-domain that was seen to decrease in a manner parallel to the individual N-domain in BL-N (Figure 6B). The observed disappearance of labeled protein from the soluble fraction of *E. coli* was in each case attributable to the formation of inclusion bodies rather than to proteolysis, given that no significant decrease in label was observed when whole cells were analyzed in the same manner (data not shown). Therefore, the N- and C-domains of CoA transferase are less soluble than the wild-type or hinge-mutant forms of the enzyme *in vivo*; once again, the C-domain is less soluble than the N-domain under these conditions.

Conformational Changes in the N- and C-Domains upon Assembly to the Active Enzyme Form. The folding of a polypeptide to its native state generally involves a progressive compaction due to the formation of secondary, tertiary, and quaternary structures [reviewed in Jaenicke (1991), Landry and Gierasch (1994), and Miranker and Dobson (1996)]. Since our preliminary results suggested that the purified N- and C-domains initially lack a native fold, we probed for conformational rearrangements associated with their assembly to the active enzyme form. For these studies, a sample of the N_2C_2—domain complex was generated and purified as described in Experimental Procedures. The specific activity of this purified sample was 17 μmol/min/mg, equivalent to the level of wild-type enzyme.

Far-ultraviolet circular dichroism (far-UV CD) spectra were recorded to estimate the secondary structure of the various engineered forms of CoA transferase. The spectrum recorded for the purified N-domain was characterized by a maximum near 195 nm and a minimum near 215 nm, most likely reflecting significant β-sheet and/or β-turn components (Figure 7A). The spectrum recorded for the purified C-domain was characterized by a maximum and minimum displaced to wavelengths lower than those of the N-domain spectrum, reflecting a mixture of random-coil and β-structure (Figure 7A). The secondary structure of each purified domain was estimated to be only about 20% α-helical, similar to the value predicted for the wild-type enzyme and the hinge-mutant. The sum of the individual N- and C-domain spectra was clearly distinct from the profile recorded for an equimolar mixture of the two domains following incubation at 22 °C (Figure 7B). Moreover, the latter spectrum was very similar to that determined for the wild-type enzyme or the hinge-mutant (Figure 7B). The relationships between the observed and summed spectra were emphasized by the corresponding difference spectra, each of which revealed a strong positive signal near 200 nm and a much weaker negative signal near 220 nm (Figure 7C). These results are consistent with the productive assembly of the N- and C-domains being associated with a conversion of random coil to β-structure (Murray et al., 1969; Woody, 1995). Finally, the sum of the spectra recorded for the individual domains was very similar to the profile determined for a mixture of the domains, which was preincubated at 4 °C instead of 22 °C. These results suggest that (1) the two domains do not assemble to form a complex at 4 °C and (2) they assume an overall secondary structure similar to that of wild-type CoA transferase when they assemble after incubation at a permissive temperature.

Given that the N- and C-domains contain one and two tryptophan residues (respectively), measurements of intrinsic fluorescence were carried out in order to study further the conformational rearrangements associated with the assembly

Table 1: Fluorescence Properties of Engineered CoA Transferase Proteins

sample[a]	$\lambda_{max,em}$[b] (nm)	K_{S-V} (acrylamide)[c] (M^{-1})
wild-type		
native	335.1	3.82 ± 0.22[d]
6 M GuHCl	346.2	
hinge-mutant		
native	335.4	3.46 ± 0.09[d]
6 M GuHCl	346.5	
N-domain		
native	338.1	8.41 ± 0.30[e]
6 M GuHCl	346.0	
C-domain		
native	342.6	10.30 ± 0.08[e]
6 M GuHCl	346.6	
domain mixture[f]		
native	335.4	3.64 ± 0.06[e]
6 M GuHCl	346.2	
tryptophan	348.0	19.46 ± 0.18[e]

[a] Protein samples were prepared as 0.07 mg/mL solutions in 0.2 M KH_2PO_4, 0.1 mM EDTA, 10 mM 2ME (pH 7.4). A standard solution of tryptophan (1.8 mg/mL) was prepared using the same buffer. [b] Wavelength of maximal tryptophan fluorescence; excitation at 295 nm. [c] Stern-Volmer constant for quenching by acrylamide, according to the relation: $F_0/F = 1 + K_{S-V}$[acrylamide]. [d] Average of three experiments; error is sample standard deviation. [e] Average of two experiments; error is deviation from mean. [f] Equimolar mixture of the N- and C-domains.

of the domains to the active enzyme form (Table 1). Excitation at 295 nm produced a wavelength of maximal fluorescence ($\lambda_{max,em}$) of 338 nm with the N-domain and 343 nm with the C-domain. These maxima were significantly red-shifted relative to the $\lambda_{max,em}$ value of 335 nm determined upon excitation at 295 nm of the wild-type enzyme, hinge-mutant, or preassembled N- and C-domains (Table 1). Moreover, the $\lambda_{max,em}$ determined for the purified C-domain most closely resembled the $\lambda_{max,em}$ value of 346 nm recorded for each of these proteins in 6.0 M GuHCl (Table 1). The intrinsic tryptophan fluorescence of each polypeptide was further assessed by measuring its sensitivity to quenching by acrylamide, a property reflected by the magnitude of K_{S-V}, the Stern−Volmer constant (Martin et al., 1991). The K_{S-V} estimates revealed that the intrinsic fluorescence of the purified N- and C-domains was more sensitive to acrylamide quenching than that of the wild-type enzyme, hinge-mutant, or preassembled complex (Table 1). Moreover, the K_{S-V} value determined for the C-domain was closest to that calculated for free tryptophan in solution (Table 1), consistent with the fluorescence spectra described above. Together, these results suggest that (1) the tryptophan residues of the purified domains are more exposed to solvent than those of the wild-type enzyme and (2) the tryptophan residues of the purified C-domain are, on average, more accessible to solvent than that of the N-domain.

Lastly, we investigated the quaternary structure of the two purified domains and the complex produced by their mixing and subsequent incubation. Sedimentation equilibrium and light-scattering studies revealed that the individual N- and C-domains are dimeric and monomeric, respectively, in benign buffer (Figure 8 and Table 2). Similar results were obtained from SDS−PAGE analysis of the purified domain polypeptides treated with the cross-linking reagents BS^3 or DTSSP (data not shown). Sedimentation-equilibrium and light-scattering studies (Table 2) indicated that the hinge-mutant and the complex assembled from the isolated domains

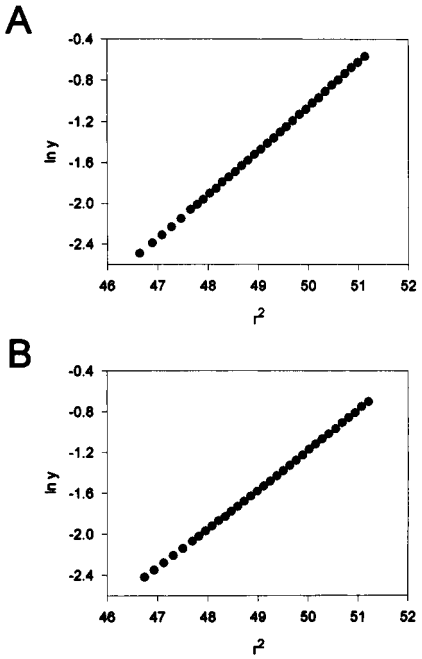

FIGURE 8: Molecular weight determinations by sedimentation equilibrium. Apparent weight−average molecular weights of 5.32×10^4 for the N-domain in panel A and 2.46×10^4 for the C-domain in panel B were determined as described in Experimental Procedures. Note that both ln y vs r^2 plots are linear, indicating that neither of the two domain polypeptides undergoes association or dissociation at the protein concentrations examined.

Table 2: Quaternary Structure of Engineered CoA Transferase Proteins

protein	M_w ($\times 10^4$)[a]	M_w ($\times 10^4$)[b]	4° structure[c]
wild-type[d]	9.15	ND[e]	$(N\sim C)_2$
hinge-mutant	9.22	11.1	N_2C_2
N-domain	5.32	ND	N_2
C-domain	2.46	2.52	C
domain mixture[f]	ND	10.2	N_2C_2

[a] Weight-average molecular weight determined by sedimentation equilibrium. [b] Weight-average molecular weight determined by light scattering. [c] Quaternary structure: N, N-domain; C, C-domain; and ∼, hydrophilic linker. [d] Data taken from White and Jencks (1976). [e] ND, not determined. [f] Equimolar mixture of the N- and C-domains.

contain two of each domain (N_2C_2). This was consistent with previous sedimentation-equilibrium analyses of the wild-type enzyme by White and Jencks (1976), who proposed that this enzyme is a homodimer consisting of two N-domains and two C-domains. In a parallel experiment, equimolar mixtures of the N- and C-domains incubated at 22 °C for 3 h or maintained at 4 °C were analyzed by PAGE under nondenaturing conditions (Figure 9). The major species in the mixture at 22 °C was a complex with electrophoretic properties identical to those of the preassembled hinge-mutant (Figure 9). In contrast, the two most intense bands in the mixture at 4 °C corresponded to the individual domains, while the N_2C_2−domain complex was a relatively minor background component (Figure 9). Thus, when mixed and incubated at a permissive temperature, one dimeric N-domain complex and two monomeric C-domains associate to yield an active N_2C_2 species.

Refolding of CoA Transferase from Denaturant. If (1) an intact hinge region is not required for the folding of CoA transferase *in vivo* and (2) the purified N- and C-domains are capable of assembling *in vitro* under benign conditions,

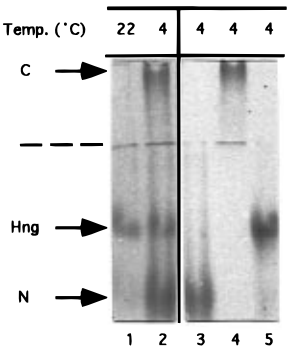

FIGURE 9: Analysis of the assembly of the N_2C_2−domain complex by PAGE under nondenaturing conditions. The purified N- and C-domains (100 μM each) were mixed and incubated at 22 °C for 3 h (lane 1) or maintained at 4 °C (lane 2). The purified N-domain (lane 3), C-domain (lane 4), and hinge-mutant (lane 5) were included as standards. All protein samples were subjected to electrophoresis under nondenaturing conditions through a 3% (w/v) stacking gel (pH 6.8) followed by a 6% (w/v) resolving gel (pH 8.8), both of which were subsequently stained with Coomassie-Blue. Bands representing the C-domain (C), N_2C_2 complex (Hng), and dimeric N-domain (N) are indicated by appropriately labeled arrows. The dashed line indicates the interface between the stacking and resolving gels. The amount of protein loaded per lane was 7.5 μg (lane 1), 20 μg (lane 2), and 10 μg (lanes 3−5).

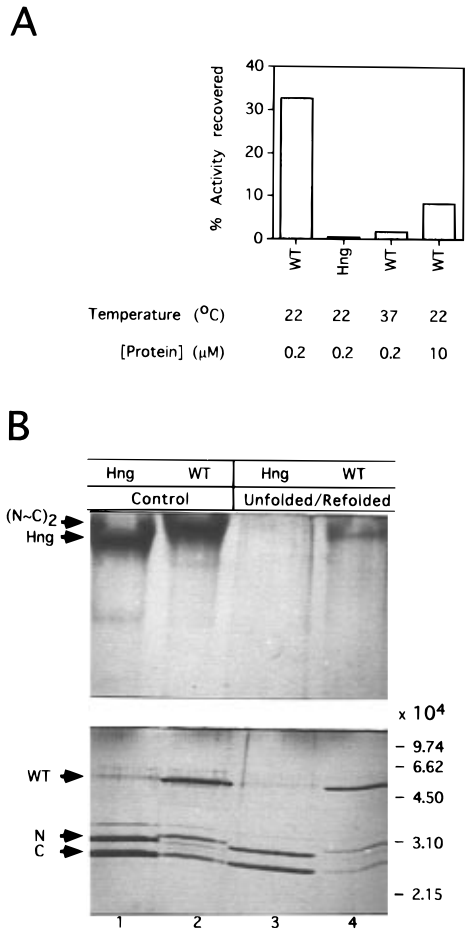

FIGURE 10: Refolding of CoA transferase *in vitro*. The wild-type enzyme (WT) and hinge-mutant (Hng) were denatured in 6.0 M GuHCl at 22 °C for at least 5 h before dilution (50-fold) in refolding buffer. The final protein concentration was 0.2 μM of wild-type monomer or of each domain of the hinge-mutant in the refolding solution. (A) Yield of active enzyme recovered upon renaturation (expressed as a percentage of the initial activity of nondenatured enzyme). The refolding temperature and protein concentration are indicated in each case. (B) Analysis of diluted wild-type CoA transferase or hinge-mutant by PAGE under nondenaturing conditions (top) and SDS−PAGE (bottom). Lanes 1 and 2, control samples diluted without prior denaturation; lanes 3 and 4, samples diluted after unfolding in 6.0 M GuHCl. For both gels, 1 μg of control or refolded protein was loaded per lane. Bands representing the wild-type enzyme [(N∼C)$_2$], hinge-mutant (Hng), 5.2×10^4 wild-type subunit (WT), 2.7×10^4 N-domain (N), and 2.4×10^4 C-domain (C) are indicated by appropriately labeled arrows. The gels were stained with $AgNO_3$; the native gel consisted of a 3% (w/v) stacking gel (pH 6.8) followed by a 9% (w/v) resolving gel (pH 8.8).

then the fully unfolded hinge-mutant form of CoA transferase should be capable of refolding from denaturant *in vitro*. To test this hypothesis, we determined the activity recoverable from each of the hinge-mutant and wild-type forms of the enzyme upon its dilution from a solution containing 6.0 M GuHCl. At this concentration of denaturant, both forms of CoA transferase were equally devoid of secondary and tertiary structure, as proven by far-UV CD and fluorescence measurements (data not shown). The wild-type enzyme (final concentration of monomer, 0.2 μM) refolded spontaneously at room temperature with yields of 35% of the initial activity (Figure 10A). Conversely, the hinge-mutant was found not to refold under the same conditions (Figure 10A). Analysis of the diluted samples by SDS−PAGE revealed that the wild-type polypeptide was partially nicked (Figure 10B), implying that the yield of refolded enzyme was most likely an underestimate. Only the diluted wild-type enzyme was detected by PAGE under nondenaturing conditions, indicating that the hinge-mutant aggregated following its dilution into refolding buffer (Figure 10B). Finally, experiments performed at either a higher protein concentration (10 μM instead of 0.2 μM) or a higher temperature (37 °C instead of 22 °C) revealed that the refolding of active, wild-type enzyme was compromised by conditions which promote the aggregation of non-native polypeptides (Figure 10A). From these results, it is suggested that the presence of the hydrophilic linker may enable the wild-type enzyme to compete more effectively than the hinge-mutant with off-pathway aggregation reactions during its refolding *in vitro*.

DISCUSSION

Folding and Assembly of the N- and C-Domains. To determine whether the N- and C-domains fold independently, we have examined the expression of a hinge-mutant of this enzyme, designed by introducing a stop codon and a reinitiation site in the portion of the cDNA encoding the hydrophilic linker. Although a strong Shine−Dalgarno sequence is properly situated upstream of this reinitiation site (Shine & Dalgarno, 1974; Gold & Stormo, 1987, 1990; Schoner et al., 1990; de Boer & Hui, 1990), synthesis of the C-domain was only 20% as efficient as that of the N-domain encoded by the same operon, based on SDS−PAGE analyses of whole BL-Hng cells grown in the presence of 0.1 mM IPTG (data not shown). The low efficiency of translation is reasonable in this case, given that low yields of protein synthesis are commonly observed with reinitiation sites created by site-directed mutagenesis (Gold & Stormo, 1987). Despite this inefficiency of translation, equimolar amounts of N- and C-domain polypeptides did associate in the BL- and HB-Hng strains to generate an adduct with levels of enzyme activity and an oligomeric structure equivalent to those of the wild-type enzyme. An active enzyme complex was also observed in lysates recovered from HB-N+C and

HB-C/N. Thus, sequestering the genes which encode the N- and C-domains on separate plasmids or downstream of separate promoters on the same plasmid did not preclude the formation of active CoA transferase in *E. coli*. We infer from these results that the two domains of CoA transferase need not be synthesized in proximity to produce a correctly folded enzyme. Comparable results involving the assembly *in vivo* of two complementary fragments derived from mitochondrial creatine kinase have recently been reported (Gross et al., 1996).

The purified N- and C-domains assembled to form an active enzyme complex *in vitro* under nondenaturing conditions. This complex could only be generated from domains purified in the presence of 2ME, suggesting that the conformation of one or both domain polypeptides is sensitive to undesirable side reactions involving the oxidation of cysteine residues. In addition, the initial rate of assembly increased as the incubation temperature was raised to a maximum of 22 °C, above which competing aggregation reactions became significant (see below). Obviously, this increase in the rate of association was partially due to the enhanced rate of diffusion of the two domains. However, the absence of any increase in activity in mixtures maintained at 4 °C suggests that a minimum of kinetic energy may also be required for a conformational change that accompanies the assembly of the two domains to the active enzyme form. This conclusion is consistent with the large E_a value [$(2.9 \pm 0.4) \times 10^2$ kJ/mol)] determined for the production of CoA transferase activity. This high E_a barrier is reasonable when compared with those values reported for the folding of other enzymes, including: 112 kJ/mol for octopine dehydrogenase (Zettlmeissl et al., 1984); 113 kJ/mol for α-lytic protease (Baker et al., 1992); and 172 and 240 kJ/mol for the tetrameric lactate dehydrogenase isoenzymes from heart and muscle, respectively (Rudolph et al., 1977). Consistent with the magnitude of these values, we predict that structural rearrangements associated with folding should be more complex (and the corresponding E_a barriers greater) for multimeric enzymes such as CoA transferase and lactate dehydrogenase than for smaller, monomeric enzymes such as octopine dehydrogenase and α-lytic protease. That enzyme activity appeared with apparent first-order kinetics in equimolar mixtures of the domains over a range of protein concentrations (0.01−0.1 mM N- or C-domain) supports the hypothesis that a conformational rearrangement is required in addition to domain pairing for the production of an active enzyme complex. Significantly, the rate of accumulation of enzyme activity was directly proportional to the concentration of the domains in each mixture examined. Also, maximal enzyme activity was greatest in the N- and C-domain mixtures of highest protein concentration. These data may be rationalized in the following manner: (1) a rapid equilibrium exists between the separate domains and an initial assembled complex (see Figure 11A and below), (2) the rate of formation of this initial complex is predicted to be much less than its rate of dissociation, and (3) this rapid equilibrium is followed by a relatively slow, nearly irreversible conformational change to yield active CoA transferase. A similar mechanism has been described for the reversible association of enzyme with substrate (Fersht, 1985) or slow-binding inhibitor (Morrison & Walsh, 1988).

Thermal Instability of the N- and C-Domains. The purified N- and C-domains were found to be more susceptible to

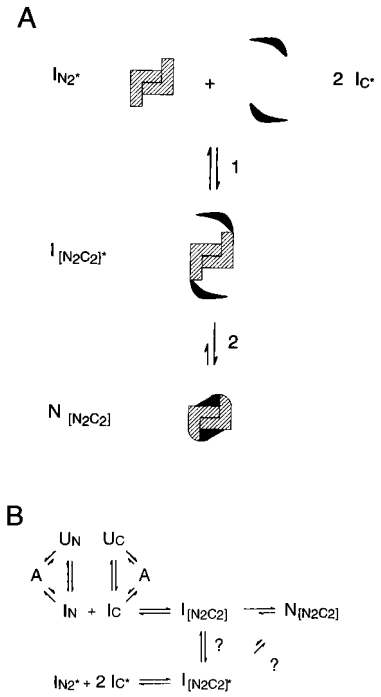

FIGURE 11: Model describing the folding properties of CoA transferase. (A) Assembly of the N- and C-domains to the active enzyme form. (1) Each of two partially folded, interacting N-domains (I_{N2^*}) associates with an incompletely folded C-domain (I_{C^*}). This docking event is fast relative to the ensuing rearrangement in secondary and tertiary structure (2), which converts the initial assembly intermediate ($I_{[N2C2]^*}$) to the native enzyme ($N_{[N2C2]}$) and may involve additional intermediates. (B) Folding and assembly from two initial states. Firstly, the denatured N- and C-domains (U_N and U_C, respectively) convert to early intermediate forms (I_N and I_C), which may then associate incorrectly to yield aggregates (A) or correctly to produce an intermediate adduct ($I_{[N2C2]}$) followed by the native enzyme form ($N_{[N2C2]}$). Aggregation occurs predominantly if the domains are not covalently linked. Secondly, each domain achieves a metastable fold when produced in *E. coli*, yielding a second class of intermediates (I_{N2^*} and I_{C^*}). Whether or not they are covalently linked to each other, these partially folded N- and C-domains may then assemble to form a second intermediate complex ($I_{[N2C2]^*}$). Finally, $I_{[N2C2]^*}$ converts to $N_{[N2C2]}$, either directly or indirectly through $I_{[N2C2]}$. Regardless of the point at which folding is initiated, productive interactions between the N- and C-domains are essential for stabilizing the native enzyme form ($N_{[N2C2]}$).

aggregation *in vitro* and *in vivo* than the wild-type enzyme or hinge-mutant, which further suggests that the individual domains lack stable, compact structures. The temperature-shift experiments indicated that the ability of the domain polypeptides to assemble to the active enzyme form was compromised following their preincubation at 37 °C. The resulting decrease in the yield of active CoA transferase coincided with the appearance of a precipitate, and may therefore be attributed to the aggregation of the domains. Furthermore, higher levels of enzyme activity were recovered from mixtures diluted prior to their preincubation at 37 °C (data not shown), consistent with the observation that aggregation occurs more readily in solutions of higher protein concentration (Jaenicke, 1982, 1991; Kim & Baldwin, 1982; Goldberg et al., 1991; van der Vies et al., 1992). The decrease in recoverable enzyme activity was best described by a double-exponential equation, which suggests that the rate of aggregation may be limited by the unfolding of the individual N- and C-domains in rapid equilibrium with an initial assembled complex. Importantly, the minimal specific enzyme activity recovered even after lengthy preincubation

at 37 °C never reached zero (Figure 5). This suggests the existence of a subpopulation within the N- and C-domain mixture consisting of conformers capable of associating and adopting a stable native fold immediately (see below). Moreover, this minimal recoverable activity may reflect the background enzyme observed at 4 °C and detected by nondenaturing PAGE (Figure 9, lane 2). As revealed by the pulse-chase experiments, the individual domains also formed inclusion bodies much more readily than the wild-type enzyme or the C-domain of the hinge-mutant in *E. coli*. The disappearance of label was also best described by double-exponential equations, suggesting that the aggregation of the various CoA transferase polypeptides *in vivo* may proceed through complex unfolding pathways consisting of more than one first-order, partially rate-limiting component.

The aggregation of proteins is generally attributed to unproductive intermolecular interactions involving transiently exposed hydrophobic surfaces (Jaenicke, 1982, 1991; Kim & Baldwin, 1982; Zettlmeissl et al., 1984). These nonpolar regions may no longer be buried in the purified N- and C-domains because the latter are misfolded or only partially folded. Alternatively, the N- and C-domain polypeptides may be susceptible to aggregation, despite possessing a native-like fold, due to exposure of hydrophobic patches which are normally sequestered in the interdomain interface of the wild-type enzyme. Results from far-UV CD and fluorescence measurements suggest that the purified C-domain possesses a relatively open, unfolded structure, whereas the fold of the purified N-domain may be more native-like (see below). This prediction is supported by our observation that the C-domain was consistently more insoluble than the N-domain, both *in vitro* and *in vivo*. Thus, we propose that the relative ease with which the C-domain was found to aggregate may be due to its lack of a compact, native-like fold.

Structural Rearrangements Concomitant with Assembly to the Active Enzyme Form. The association *in vitro* of the individual N- and C-domains to form active enzyme involved substantial conformational changes. Far-UV CD analyses indicated a significant amount of β-sheet or β-turn (N-domain) and random coil (C-domain). The overall secondary structures of the two domains were altered following their assembly to the active enzyme form. Although the exact nature of this conformational change could not be assigned from the difference CD profile alone (Figure 7C), the presence of a positive signal near 200 nm on this spectrum reflects a reorganization of random-coil upon formation of the active complex. This rearrangement may reasonably be attributed for the most part to the C-domain. The measurements of intrinsic tryptophan fluorescence in the presence and absence of the collisional quencher acrylamide indicated that the tryptophan residues of the N- and C-domains were more buried following the assembly of the domains to produce active CoA transferase. These results suggest, as one possibility, that both domains acquire a more compact structure following their association. Alternatively, the tryptophan residues may be sequestered from solvent in the wild-type enzyme simply by being located at the interface between the N- and C-domains. However, it may be reasonably inferred from the combination of CD and fluorescence data that at least the C-domain possesses a more open, unfolded structure as a purified polypeptide rather than as a properly assembled component of the wild-type enzyme. Finally, ultracentrifugation, light-scattering, and nondenaturing PAGE showed that the purified N-domain is dimeric (N_2), the purified C-domain is monomeric, and the active enzyme complex consists of two molecules of each domain polypeptide (N_2C_2). The ln y versus r^2 plots derived from the sedimentation-equilibrium analyses were linear, indicating that no significant association-dissociation equilibria occurred at the protein concentrations examined. These results can be interpreted according to a simple model (Figure 11A) in which each of two partially folded, interacting N-domains associates with an incompletely folded C-domain. This docking event is followed by a much slower conformational change, most likely involving (1) a rearrangement in secondary and tertiary structure, leading to the stabilization and compaction of both domains and (2) the exclusion of water and the burial of hydrophobic sidechains at the interface between the N- and C-domains [reviewed in Jaenicke (1987) and Price (1994)]. These processes ultimately stabilize the native fold of the enzyme. Our data do not rule out, however, that the dimer interface of the N_2 species is different from that in the N_2C_2 complex. In other words, the dimerization of the N-domain may involve inappropriate intermolecular interactions, analogous to those described for cytoplasmic malate dehydrogenase (Rudolph et al., 1986), the homodimeric form of the bacterial luciferase β-subunit (Sinclair et al., 1994), or various mutant, oligomeric forms of the phage P22 coat protein (Teschke & King, 1995). If this were true, then the active complex might be assembled by a more complicated mechanism, involving perhaps the preliminary dissociation of the N-domains from the N_2 dimer or a reshuffling of the N_2 interface subsequent to the formation of an early N_2C_2 complex.

Similar models have been described for the association of complementary fragments derived from other proteins, including barnase (Kippen et al., 1994; Kippen & Fersht, 1995), chymotrypsin inhibitor-2 (Prat Gay et al., 1994; Prat Gay & Fersht, 1994a,b; Ruiz-Sanz et al., 1995), creatine kinase (Gross et al., 1996), tryptophan repressor (Tasayco & Carey, 1992; Wu et al., 1994), cytochrome *c* (Wu et al., 1994), and phosphoglycerate kinase (Pecorari et al., 1993). In each of these examples, the assembly of the complementary fragments is accompanied by significant structural rearrangements. The formation of the complex need not precede these adjustments, but may instead result merely from the association of native structures in equilibrium with predominantly non-native conformers. In studies on barnase (Ruiz-Sanz et al., 1995), this question has been addressed by evalulating the effect of a series of mutations on the stability and the association kinetics of complementary fragments. This type of analysis, which leads to a description of the structure of the transition state for complex formation, could in the future be applied to the N- and C-domains of CoA transferase. Our present results do suggest, however, that the purified domains consist of several species at different stages of folding, each possessing a different ability to assemble to yield active enzyme. The presence at "zero" time of minimal background activity recoverable from the N- and C-domain mixtures implies that some conformers can associate to form active CoA transferase immediately. The yield of CoA transferase activity obtained at 22 °C decreases with the lengthy storage of the purified domains, suggesting that yet other conformers incapable of productive assembly accumulate with time. On the basis of this study,

we postulate that the predominant conformers in the N- and C-domain preparations are capable of forming active CoA transferase by an assembly event which also involves significant structural rearrangements.

Implications for the Folding Pathway of CoA Transferase. We have shown that the hydrophilic linker is essential for the recovery of CoA transferase activity upon dilution of the enzyme from denaturant. A similar role has been attributed to the linker region of lactate dehydrogenase (Opitz et al., 1987; Jaenicke, 1991). This result is not consistent with our observation that an intact hinge region is not required for the folding of mature CoA transferase in *E. coli*. We propose that chaperone systems and/or cotranslational folding events may inhibit the aggegation of the hinge-mutant within the bacterial cytosol, as implied from our recent demonstration that the spontaneous folding of CoA transferase *in vitro* is abolished by GroEL in the absence of ATP (Rochet, J.-C., unpublished material). The low refolding yield of the hinge-mutant must also be reconciled with the association of the isolated N- and C-domains to produce active enzyme *in vitro* (Figure 11B). Importantly, following their dilution, the domains of the denatured hinge-mutant were found to aggregate at a concentration 500 times less than that of the purified, mixed domains. From these results, we predict that the conformers which aggregate during the refolding of the hinge-mutant are distinct from the partially folded, purified domains, and most likely consist of unfolded polypeptides (U_N and U_C) and early folding intermediates (I_N and I_C). An intermediate adduct ($I_{[N2C2]}$) may result from the assembly of I_N and I_C, given that the latter are less susceptible to aggregation when they are covalently linked (Figure 11B). In contrast, the domains purified from *E. coli* may be altogether distinct intermediates (I_{N2*} and I_{C*}) which occur on a separate pathway (Figure 11B). Off-pathway conformers of a similar type have been described for the α- and β-subunits of bacterial luciferase produced individually in *E. coli* (Baldwin et al., 1993; Ziegler et al., 1993). Folding from I_{N*} and I_{C*} requires their association to form a second intermediate complex ($I_{[N2C2]*}$), which then converts in a rate-limiting manner to $N_{[N2C2]}$. Although the data are consistent with this model, other more complex explanations cannot be ruled out. For instance, I_{N*} and I_{C*} may not belong to an entirely separate, parallel pathway with respect to that of I_{N1} and $I_{C1,}$ but may instead also proceed through $I_{[N2C2]}$. Such a mechanism would be consistent with the general concept of proteins folding according to a framework model in which the number of intermediate states decreases progressively as the native conformation is approached [reviewed in Jaenicke (1991), Landry and Gierasch (1994), and Miranker and Dobson (1996)]. In addition, the unfolded and intermediate forms described above most likely represent an ensemble of states. A more detailed analysis of the structures of the purified N- and C-domains and of kinetic and/or equilibrium intermediates along the folding pathway of CoA transferase will be required to address these questions. It is clear, however, that the N- and C-domains of CoA transferase do not achieve their native folds completely independently of each other, but rather, through productive interactions during their folding and assembly.

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