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β-Ketoacyl-[Acyl Carrier Protein] Synthase I of *Escherichia coli*: Aspects of the Condensation Mechanism Revealed by Analyses of Mutations in the Active Site Pocket†

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ABSTRACT: β-Ketoacyl-[acyl carrier protein (ACP)] synthase forms new carbon−carbon bonds in three steps: transfer of an acyl primer from ACP to the enzyme, decarboxylation of the elongating substrate and its condensation with the acyl primer substrate. Six residues of *Escherichia coli* β-ketoacyl-ACP synthase I (KAS I) implicated in these reactions were subjected to site-directed mutagenesis. Analyses of the abilities of C163A, C163S, H298A, D306A, E309A, K328A, and H333A to carry out the three reactions lead to the following conclusions. The active site Cys-163 is not required for decarboxylation, whereas His-298 and His-333 are indispensable. Neither of the histidines is essential for increasing the nucleophilicity of Cys-163 to enable transfer of the acyl primer substrate. Maintenance of the structural integrity of the active site by Asp-306 and Glu-309 is required for decarboxylation but not for transfer. One function of Lys-328 occurs very early in catalysis, potentially before transfer. These results in conjunction with structural analyses of substrate complexes have led to a model for KAS I catalysis [Olsen, J. G., Kadziola, A., von Wettstein-Knowles, P., Siggaard-Andersen, M., and Larsen, S. (2001) *Structure 9,* 233−243]. Another facet of catalysis revealed by the mutant analyses is that the acyl primer transfer activity of β-ketoacyl-ACP synthase I is inhibited by free ACP at physiological concentrations. Differences in the inhibitory response by individual mutant proteins indicate that interaction of free ACP with Cys-163, Asp-306, Glu-309, Lys-328, and His-333 might form a sensitive regulatory mechanism for the transfer of acyl primers.

The β-ketoacyl-ACP[1] synthase or condensing enzyme component of fatty acid and polyketide synthases creates new carbon−carbon bonds by carrying out Claisen condensations. Such reactions, resulting in elongation of specific primers, are tripartite: (i) transfer of a phosphopantotheine-bound primer to a cysteine residue of the condensing enzyme; (ii) decarboxylation of an acyl carrier bound donor unit to give a carbanion; and (iii) condensation of the carbanion with the carbonyl carbon of the enzyme-bound primer. To synthesize their membrane fatty acids, *E. coli* cells use three KAS enzymes. Although they carry out some of the same extension steps, KAS III specializes in the initial elongation reaction, while KAS I is unique in carrying out the first and KAS II the last step of the unsaturated pathway, respectively. Such substrate specificities of KAS enzymes result in an extraordinarily diverse range of carbon chains that give rise to a myriad of different fatty acids and polyketides. This has stirred the interest of the plant oil and pharmaceutical biotechnologists with their goals of producing new fatty acids and antibiotics. Such objectives would be assisted by a detailed picture of the reaction mechanism.

The identity of the active site cysteine was disclosed 13 years ago (*1*, *2*), and a model for catalysis was put forth in 1993 implicating several basic residues: His-298, Lys-328, and His-333 (*3*). Attention was also drawn to two other of the conserved residues, Asp-306 and Glu-309. Further advances have been made possible with the solving of the crystal structures of *E. coli* KAS I, KAS II, and KAS III as well as that for a CHS from alfalfa (*4*−*9*). These structures reveal dimeric enzymes having highly similar core domains based on the α-β-α-β-α fold, but differing in their capping domains and entrances to the active site. The active site residues in KAS III and CHS differ from those noted above

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[1] Abbreviations: Ac-ACP, acetyl-ACP; ACP, acyl carrier protein; CIPE, cerulenin-inhibited protein extract; CHS, chalcone synthase; CLF, chain length factor; DTT, dithiothreitol; FAS, fatty acid synthase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; KAS, β-ketoacyl-[acyl carrier protein] synthase; Mal-ACP, malonyl-ACP; MCAT, malonyl-coenzyme A:acyl carrier protein transacylase; Ni-NTA, nickel-nitrilotriacetic acid; PKS, polyketide synthase; TLS, thiolase.

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for KAS I and KAS II in that an asparagine replaces His-333 and an analogue of Lys-328 is missing. We have proposed that KAS I and KAS II enzymes with an active site triad consisting of cysteine-histidine-histidine (CHH) carry out the decarboxylation step by a different mechanism than KAS III and CHS enzymes with a cysteine-histidine-asparagine (CHN) triad (*10*). The α-β-α-β-α fold was first described for a degradative thiolase (TLS) from *Saccharomyces cerevisiae* (*11*) and is also characteristic for the biosynthetic TLS from *Zoogloea ramigera* (*12*). The Claisen condensation carried out by TLS enzymes differs from that described above in lacking the decarboxylation step (*11*). Instead the carbanion is created by abstraction of a proton from the elongating substrate by a second cysteine. The asparagine replacing His-298 has not been assigned a primary function in catalysis although it is part of a hydrogen bond network.

The architectures of KAS I complexed with the inhibitor thiolactomycin, of KAS I and KAS II with the inhibitor cerulenin, and of KAS I with C10 and C12 fatty acyl substrates have also been obtained (*10*, *13*, *14*). Analyses of the inhibitor complexes lead to the suggestion that they simulate binding of substrates in the active site: thiolactomycin in place of malonyl, and cerulenin in place of the acyl chain. Small differences in the active sites compared to the noncomplexed enzyme were observed, notably of His-298 (*14*). In the C10 and C12 acyl substrate complexes, however, analogous movement of His-298 does not take place (*10*). Very recently our knowledge of the decarboxylation reaction has been significantly extended by analyzing a series of mutants of the active site cysteine in the condensing domain of mammalian FAS (*15*). Replacement of this residue with glutamine turned the enzyme into a very efficient decarboxylase, far more so than when iodoacetamide is bound to the active site cysteine. Combined with additional biochemical analyses and modeling of glutamine into the *E. coli* KAS II crystal structure, a plausible structural molecular explanation was adduced. Namely, the carbonyl group of the glutamine was able to mimic that of an acyl primer or alkyl inhibitor such as iodoacetamide and induce the same conformational shift altering the balance between the condensation and decarboxylation reactions envisaged by Lynen's group 24 years ago (*16*). By comparison to the just noted site-directed mutagenesis experiments, those carried out on KAS III from *Cuphea wrightii* (*17*), for which the crystal structure is unknown, resulted in a number of observations that are difficult to reconcile with the models for catalysis emerging from the studies with the *E. coli* enzymes referred to above. Additional experiments as well as the crystal structure of this enzyme are required to reveal the bases for these differences.

In the present paper we have mutated the six residues specified above in *E. coli* KAS I and compared the ability of the wild-type and mutant enzymes to carry out the three KAS reactions. The results confirming initial observations (*18*) that all these residues are necessary for catalysis and revealing at which step they function contributed to development of a model for KAS I catalysis (*10*). We focus on a hitherto almost totally unexplored facet of the catalytic mechanism, namely, the transfer reaction in which KAS I is both substrate and enzyme. This has revealed that transfer is inhibited by the ACP released during the reaction.

MATERIALS AND METHODS

Materials. Restriction enzymes were from Boehringer Mannheim, Pfu polymerase was from Stratagene, and plasmid purification kits plus Ni-NTA Superflow resin were from Qiagen. Primers containing the mutation(s) of interest were synthesized by Operon. Ampicillin, kanamycin, and ACP came from Aldrich-Sigma. Millex-GV 200 nm and PVDF Immobilon membranes were purchased from Millipore, while the column packings Mono-Q for anion exchange and octyl Sepharose for affinity chromatography were from Pharmacia. [^{3}H]Myristic acid, [1-^{14}C]palmitic acid, and [2-^{14}C]malonyl-CoA were supplied by New England Nuclear, 4−15% gradient gels plus IPTG and high-range prestained SDS−PAGE protein standards were from Bio-Rad, and the scintillation fluid OptiPhase 'HiSafe3' was from Fischer Chemicals. The *E. coli* malonyl-CoA:ACP transacylase (MCAT) gene cloned in the expression vector pET11d (Novagen) was kindly provided by Dr. T. Stuitje, and the *E. coli* β-ketoacyl-ACP synthase I (KAS I) gene cloned in the expression vector pQE30 (Qiagen) was a much appreciated gift from Dr. K. Dehesh (*19*).

Acyl-ACP synthase was purified from *Vibrio harveyii* mutant 17 (*20*). This enzyme was then used to synthesize radiolabeled acyl-ACPs from ACP and [^{3}H]myristic acid or [1-^{14}C]palmitic acid (*20*), and the radiolabeled acyl-ACPs were purified by Mono-Q plus octyl Sepharose chromatography. The specific activities of the myristoyl and palmitoyl moieties were 10.7 and 2.06 TBq/mol, respectively. Malonyl-CoA:ACP transacylase (MCAT) was overexpressed in BL21-(DE) cells and purified from 100 mL of culture according to Novagen's recommendations.

Bacterial Strains and Plasmids. The *E. coli* strains used in this work were the K12 derivatives, XL1-Blue (*recA endA*1 *gyrA*96 *thi-1 hsdR*17 *supE*44 *relA*1 *lac*[*F′ proAB lacIqZDM*15 *tn10* (*tet* ^{r})]) (Stratagene) used for transformation and construction of the KAS I *fabB* mutants, plus M15 [pREP4] (*Nal* ^{S}, *Str* ^{S}, *Rif* ^{S}, *Lac*$^-$, *Ara*$^-$,*Gal*$^-$, *Mtl*$^-$, *F*$^-$, *RecA*$^+$, *Uvr*$^+$, *Lon*$^+$) (Qiagen) used for expression and purification of the proteins. *E. coli* was grown at 37 °C in Luria−Bertani medium or in SB medium (Qiagen). The expression vector pQE30 (Qiagen) and the repressor plasmid pREP4, encoding the β-lactamase and neomycin genes, respectively, were selected using 100 μg/mL ampicillin and 25 μg/mL kanamycin. Transcription of the cloned gene from the T5 promoter is regulated by two *lac*-operator sequences on pQE30 controlled by the *lacI* repressor constitutively expressed by pREP4. Upon addition of IPTG, the *fabB* gene encoding KAS I is expressed with the N-terminal sequence Met-Arg-Gly-Ser, a His tag of six residues, and Gly-Ser.

DNA Manipulations. To create mutations, Strategene's QuickChange Site-Directed Mutagenesis kit was used. pQE30-*fabB* DNA served as template for amplification with Pfu and the following primers plus their complements: D70A (5′-GCGCTTTATGAGCGCAGCATCCATTTATGC-3′), C163A (5′-CAGCTCCGCGGCTGCGACCTTCCGC-3′), C163S (5′-CAGCTCCGCGTCTGCGACCTTCCGC-3′), H298A (5′-CCTGAACTCCGCCGGTACTTCG-3′), D306A (5′-CGACTCCGGTTGGCGCAGTGAAAGAGCTGGC-3′), E309A (5′-GCGACGTGAAAGCGCTGGCAGCTATC-CGT-3′), K328A (5′-GCGATTTCTGCAACCGCAGCCAT-GACCGG-3′), and H333A (5′-GCCATGACCGGTGCCTC-

TCTGGGCGC-3′). The PCR products were subjected to *Dpn*I digestion to remove the template DNA and transformed into XL-1 Blue cells. To verify that candidate clones contained the desired mutations after transformation into M15 expression cells, they were completely sequenced, as was the pQE30-*fabB* template clone. Analyses revealed (i) that the pQE30-*fabB* clone contained the mutation V4A when compared to the wild-type sequence (*1*), and (ii) that in addition to the latter mutation, 75% of the candidate clones encoded the desired mutation. A new mutagenesis was then carried out using A4V DNA as template with the primers V4A (5′-GGATCCATGAAACGTGCAGTGATTACTGGCC-3′) and its complement to create the true wild-type gene. Since no differences could be detected using any of the parameters described below between the KAS I proteins expressed from pQE30-*fabB* (V4A) and true wild-type (A4V), we decided to continue with the original set of mutations introduced into pQE30-*fabB* (V4A) and designate this as wild-type herein. The double mutants C163A + H298A, C163A + D306A, C163A + E309A, C163A + K328A, and C163A + H333A were synthesized either by digesting single mutants with *Cla*I and *Sph*I and ligating together appropriate fragments or by mutagenizing DNA already carrying one of the mutations. The just described mutagenesis protocol was used with the exception that complete sequencing was carried out on the initial transformants in XL1-Blue.

Expression and Purification of Recombinant KAS Proteins. All media used for expression were supplemented with ampicillin and kanamycin. Overnight cultures of recombinant *E. coli* KAS I and KAS I mutants in M15 were pelleted, resuspended, and used to inoculate 50 mL of SB and grown to $OD_{600} \sim 1$ at 37 °C. After adding IPTG to a final concentration of 1 mM to induce expression, the cultures were grown for 2 h at 25 °C. Recombinant protein was purified at 4 °C unless noted otherwise. Cells were harvested by centrifugation (10 min at 4000 rpm) and resuspended in 1−2 mL of purification buffer: 25 mM Tris-HCl (pH 7.8), 300 mM NaCl. Cell lysis consisted of three steps: (i) Lysozyme was added to a final concentration of 1 mg/mL and the solution placed on ice for 30 min. This was followed by (ii) three cycles of freezing and thawing plus (iii) two sonications of 20 s each in the cold. The cell debris was pelleted by centrifugation, and the resulting supernatant was filtered through a Millex-GV 220 nm membrane to remove traces of cell debris, and loaded onto a Ni-NTA column. Binding and washing of the column was accomplished using purification buffer supplemented with 8.7% (w/v) glycerol and 10 mM imidazole according to Qiagen's recommendations. For elution of the His-tagged recombinant proteins, the same buffer was used except that the imidazole concentration was 200 mM. The final purified protein was subjected to ultrafiltration to remove the imidazole. This was accomplished using storage buffer [25 mM Tris-HCl (pH 7.8) containing 300 mM NaCl, 2 mM EDTA, 2 mM DTT, and 8.7% (w/v) glycerol]. Protein concentrations were determined using the BCA reagent as recommended by Pierce. Aliquots were stored frozen and diluted to suitable concentrations with storage buffer before analysis. SDS−PAGE analysis was performed by the method of Laemmli (*21*) and native gradient gel analysis as specified by BioRad.

Acyl Transferase Assay. The acyl transferase assay measures the ability of KAS I to accept, that is, transfer the labeled fatty acid from ACP to itself. We choose to synthesize and use C_{14}-ACP as it has been shown to function as a substrate for both KAS I and KAS II assays (*18, 22*). The transfer reaction was carried out at 20 °C for 10−120 min. [^{3}H]-C_{14}-KAS I and [^{3}H]-C_{14}-ACP were separated by size exclusion chromatography using a 1 × 30 cm Superdex-200 column (Pharmacia) at 4 °C. The % transfer/min is defined as: C_{14}-KAS I (cpm)/[C_{14}-KAS I (cpm) + C_{14}-ACP (cpm)]·min^{-1}. In carrying out these calculations, the residual time from injection to resin of 40 s was added to the assay time. The standard 60 μL reaction mixture contained 50 mM K-PO_4 (pH 6.8), 0.5 mg/mL thyroglobulin, 0.2 μM [^{3}H]-myristoyl-ACP (∼6000 cpm), and KAS I (0.3−24 μg). In the K328A substrate analysis, up to 59 μg of enzyme was used. After incubation, 50 μL of the reaction mixture was loaded onto the column, and the proteins were separated by a buffer containing 25 mM Tris-HCl (pH 7.8), 300 mM NaCl, 8.7% (w/v) glycerol, 2 mM EDTA, at 0.4 mL/min. Fractions of 200 μL were collected, and subjected to liquid scintillation counting using a Beckman L#1701 instrument. Total cpm recovered after a transfer reaction as [^{3}H]-myristoyl-ACP + [^{3}H]myristoyl-KAS were equivalent to the cpm recovered when [^{3}H]myristoyl-ACP was incubated in the absence of a KAS protein, that is, 70 ± 10% of the cpm injected.

Malonyl Decarboxylase Assay. The malonyl decarboxylase assay measures the ability of KAS I to decarboxylate the donor substrate malonyl-ACP, synthesized from malonyl-CoA. (i) ACP and MCAT were preincubated with dithiothreitol (DTT) for 15−60 min on ice. Synthesis of malonyl-ACP was accomplished using 25 mM K-PO_4 (pH 6.8), 12 μM ACP, 10.1 μM [^{14}C]malonyl-CoA (10 000 dpm/nmol), 1 mM DTT, and 5 nL of our MCAT preparation per assay. After 5 min at 25 °C, the reaction mixture, designated A, was transferred to ice. (ii) For a 100 μL decarboxylation reaction, 83 μL of A was aliquoted to an Eppendorf tube with 17 μL (0.1−10 μM monomer) of KAS I wild-type or mutant protein. While in initial experiments the KAS protein was incubated with 5 mM DTT before carrying out the decarboxylation reaction, this was shown to be unnecessary. After 10 min at 25 °C, the reaction was stopped by adding 800 μL of cold 10% (w/v) trichloroacetic acid (4 °C). The solution was centrifuged, and the pelleted proteins were resuspended in 10 mM Tris-HCl (pH 6.8) with the aid of scraping the sides of the Eppendorf tube; 1 M urea PAGE (13.3% acrylamide) using a gel temperature of 17 °C separated the acyl-ACPs on the basis of the chain length of the acyl substitution and the presence of functional groups (*23*). The acyl-ACPs were electroblotted for 45 min to a PVDF membrane using 50 mM H_3BO_3/NaOH (pH 8) for autoradiography using a Molecular Dynamics Storm 840 PhosphorImager.

Elongation/Rescue Assay. The elongation/rescue assay measures the ability of a purified KAS I protein preparation to restore elongation activity to a cerulenin-inhibited crude *E. coli* extract (CIPE). Cerulenin is a KAS-specific inhibitor that functions by covalently binding to the active site cysteine of KAS I and KAS II (*1, 2, 24*). The *E. coli* protein extract was made and treated with 0.5 mM cerulenin as previously described (*24*). The 100 μL reaction mixture prepared on

ice contained 10 μM ACP, 10 μM acetyl-CoA, 60.1 μM [2-^{14}C]malonyl-CoA (1670 dpm/nmol), 50 mM K-PO_4, pH 6.8 buffer, 1 mM DTT plus cerulenin-treated extract, and 0.2−0.4 μg of a purified KAS preparation. After 20 min at 37 °C, acyl-ACPs were precipitated, resuspended, and resolved by 4 M urea PAGE (13.3% acrylamide) at 17 °C for visualization as described above. A total of 1250 cpm of [1-^{14}C]palmitoyl-ACP was included on the gel as a standard.

RESULTS

DNA Manipulation and Purification of Recombinant KAS I and KAS I Mutant Proteins. The pQE30-*fabB* clone received from Dr. K. Dehesh (*19*) served as template during site-directed mutagenesis. Complete sequencing identified clones containing only the desired exchanges. Overexpression and purification of *fabB* yielded 1−4 mg of KAS His-tagged protein/50 mL of culture. The pQE expression system is stringently regulated by the T7 promoter and two copies of the highly efficient lac operator. Overexpression was carried out for 2 h at 25 °C, during which time no lethal effect of the KAS I mutations on the *E. coli* cells was observed, in contrast to the marked inhibition on growth noted for expression of KAS II (*25*). Using longer times for expression gave rise to increased total protein, but the relative amount of soluble protein decreased significantly. The resulting proteins were ca. 95% pure as estimated by Coomassie-stained SDS−PAGE (Figure 1A). All the proteins showed one single band close to 45 kDa, indicating that no significant degradation had occurred. Subjecting the proteins to native gradient PAGE (4−15%) divided them into groups. Both the mutant H298A and H333A proteins behaved exactly as wild-type, giving a single broad band of ca. 66 kDa and a weak band around 100 kDa. The C163A, C163S, and K328A proteins each showed a band close to 80 kDa, whereas the D306A and E309A proteins showed a band around 100 kDa. In addition to the specified bands, the initial gel revealed that the C163A, C163S, D306A, and E309A samples contained additional protein, not folded into a discrete form, which appears as smears at higher molecular weights than the bands. A native PAGE of four of the seven mutant proteins illustrates this (Figure 1B). Despite these differences, the crystal structures of C163A, C163S, and K328A (*10*; Olsen, unpublished results) are almost identical to that of the wild-type enzyme, inferring that the differences are not attributable to misfolding but instead to other subtle attributes of the mutated residue. These can affect protein stability under given environmental conditions, for example, those experienced in native gel electrophoresis in the present situation. The many examples of temperature-sensitive mutant proteins provide a useful parallel.

Transfer of Myristic Acid from ACP to Wild-Type and KAS I Mutant Proteins. The transfer of myristic acid from ACP to KAS I gives rise to myristoyl-KAS I and ACP. In this reaction, KAS plays the double role of enzyme and substrate, and K_{eq} = [ACP][acyl-KAS]/[KAS][acyl-ACP]. The acyl-ACP substrate and acyl-KAS product are adequately resolved on a Superdex-200 size exclusion column (Figure 2). To analyze if the transfer reaction is a true equilibrium reaction, we determined apparent equilibrium constants from the following three experiments. (i) Starting with 0.225 μM acyl-ACP and 1.1 μM KAS I and carrying out the reaction under

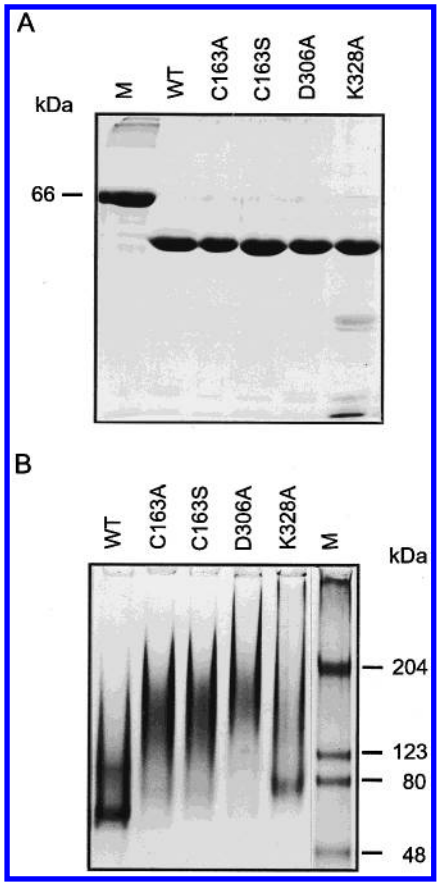

FIGURE 1: Characterization of wild-type and mutant KAS I proteins by gel electrophoresis. The His-tagged proteins were eluted from Ni-NTA columns, concentrated, and transferred to 25 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 8.7% (w/v) glycerol before subjection to electrophoresis. 6 μg of KAS I protein/lane. Gels stained with Coomassie brilliant blue. (A) A 12% SDS−PAGE of purified proteins. (B) A native gradient gel (4−15% acrylamide) of purified proteins. WT = wild-type; M = marker.

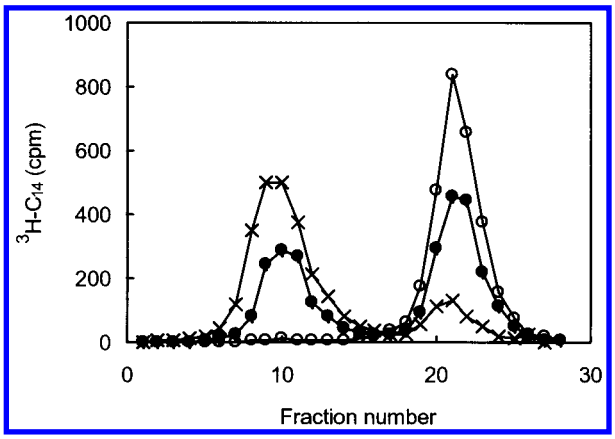

FIGURE 2: Superdex-200 column separation of C_{14}-ACP and C_{14}-KAS I wild-type and mutant proteins. Radiolabeled myristoyl-ACP was added to 7−9 μg of KAS protein in 50 mM potassium phosphate buffer and allowed to sit for 10 min at 20 °C before being injected onto the size exclusion column. The fractions were collected and subjected to liquid scintillation as detailed under Materials and Methods. C_{14}-KAS I and C_{14}-ACP elute in fractions 6−14 and 18−25, respectively. ● = wild-type; ○ = C163A; × = C163S.

conditions permitting maximum transfer resulted in 25.1% transfer of the acyl chain to KAS I, and a $K_{eq} = 1.82 \times 10^{-2}$. (ii) Starting with 0.225 μM acyl-ACP, 1.1 μM KAS I plus 0.167 μM ACP and allowing maximum transfer resulted

Table 1: Transfer Efficiency of KAS I Proteins[a]

protein	% transfer, 10 min	% transfer/μg of KAS protein		% efficiency of wild-type at 10 min
		10 min	60 min	
KAS I	42.5	6.4	6.3	100
C163S	83.9	9.2	9.1	143
H298A	39.6	4.3	3.6	67
D306A	77.5	8.8	10.0	137
E309A	32.2	5.4	−	83
K328A	30.1	4.1	13.4	63
H333A	67.8	8.7	10.1	135

[a] The % of myristic acid transferred from C_{14}-ACP to seven KAS I proteins/μg of protein was determined at 10 and 60 min. Efficiency is expressed as % of wild-type transfer. The protein concentrations which were at or close to saturation ranged from 7 to 12 μg/assay, depending on the activity of the mutant. (−) = data not available. A repeat experiment gave values of 7.6 and 7.7 at 10 and 60 min, respectively, for E309A.

in 17.5% transfer of the acyl chain to KAS I, and a $K_{eq} = 4.13 \times 10^{-2}$. (iii) Starting with 0.225 μM acyl-ACP and 1.1 μM KAS I, conditions enabling maximum transfer were employed before adding 0.167 μM ACP. The reaction was then incubated for an additional 10 min, giving 25.7% transfer and a $K_{eq} = 7.46 \times 10^{-2}$. A comparison of the first and third experiments reveals that addition of ACP at the end of the reaction has no affect since in both cases 25% transfer is obtained. By comparison, if free ACP is present during the reaction as in the second experiment, then transfer is decreased to 17%. Combined these observations imply that the transfer assay cannot be analyzed as an equilibrium reaction because the reverse reaction, transfer of the acyl chain from KAS I to ACP, does not take place at a measurable level. When the reverse reaction is negligible, the reaction goes to 100% completion. Thus, the acyl transfer reaction can be considered as formation of an ES complex, for which the initial rate can be measured as a function of one of the substrates (k_1[S]) giving half-saturation values that equal K_d (*26*). Formation of an acyl-ACP−KAS I complex is revealed by the linearity in % transfer/min from 1 to 6 μg of protein/assay (Figure 3A). The intercept with the *y* axis, 1/*k*-KAS I, was determined and found to be 1.9 μM^{-1}, indicating a binding affinity of about 5×10^{-7} M for KAS I to myristic acid (Figure 3B).

When analyzing wild-type KAS I, identical elution profiles were obtained when incubating from 10 to 120 min, as exemplified in Figure 2 and Table 1. Unless otherwise specified, the following assays were all carried out for 10 min. In Table 1, the % transfer/μg of protein for each of the investigated mutants is listed as percent of wild-type. This we designate the transfer efficiency. Inspection of the data reveals that the mutant proteins can be divided into three categories: the more efficient (C163S, D306A, H333A), the less efficient (H298A, E309A, K328A), and the nonactive (C163A). These experiments were carried out with saturating amounts of protein, which was not necessarily the same as that characterizing the wild-type protein. When carried out in the linear range as in Table 2, the same groupings occur. In this case, the transfer efficiencies of the first group were similar to that of the wild-type, while those of the second group were further reduced. The % transfer/μg of protein with respect to time was analyzed by comparing a standard assay (10 min) with one of 60 min (Table 1). Only the

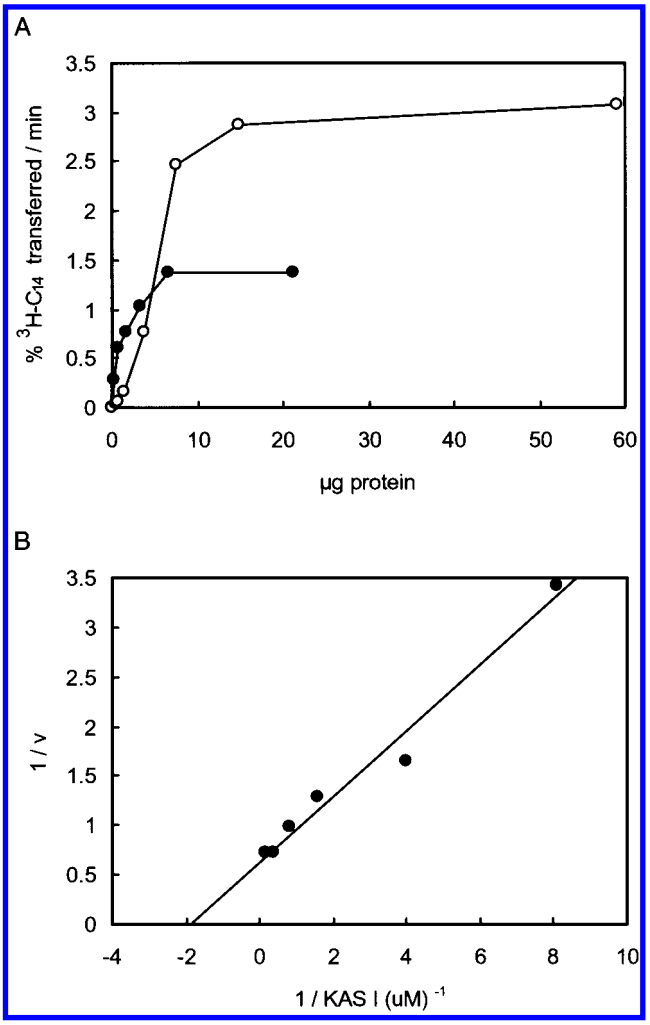

FIGURE 3: Transfer of myristic acid from ACP to wild-type and K328A KAS I proteins as a function of KAS I protein concentration. Radiolabeled myristoyl-ACP was added to KAS protein in 50 mM potassium phosphate buffer and allowed to sit for 30 min at 20 °C before being injected onto the Superdex-200 size exclusion column. Fractions were collected and subjected to liquid scintillation as detailed under Materials and Methods. (A) Substrate curves were obtained from % transfer = C_{14}-KAS I (cpm)/[C_{14}-KAS I (cpm) + C_{14}-ACP (cpm)]·min^{-1} as a function of protein concentration. (B) Lineweaver−Burke plot for the wild-type data presented in (A). v = % transfer/min; ● = wild-type; ○ = K328A.

K328A mutant KAS increased its % transfer with time, rising from 4.1% to 13.4%/μg of protein. The remaining mutants showed no significant differences in % transfer between the 10 or 60 min incubations, indicating that these mutants behaved as wild-type for 1 h. These results also indicate that once the acyl-KAS is formed it is stable. To get more information on the K328A mutant, a substrate analysis was performed (Figure 3A). In contrast to wild-type, a sigmoidal curve was obtained and the transfer continued until all the myristic acid was transferred to KAS. Analysis of the data leads to a Hill coefficient for the curve of about 3, the precise value depending on the program used, suggesting a cooperative protein with three or more binding sites. Cross-linking (*27*) and structural analyses (*10*) suggest that KAS I can exist as a tetramer. We are presently pursuing this subject.

Inhibition of Wild-Type and Mutant KAS I Proteins by Free ACP. That the observed transfer upon going to completion never exceeded 40−42% for the wild-type when working beyond the linear range (Figures 2 and 3A) implies

Table 2: Effect of the Presence of ACP on the Transfer Reaction Carried Out by Wild-Type and Mutant KAS I Proteins[a]

protein	% transfer/μg of KAS protein −ACP	% transfer/μg of KAS protein +ACP	% inhibition by ACP
KAS I	8.0	4.2	47
C163S	7.4	7.8	0
H298A	4.1	2.2	45
D306A	7.4	5.5	26
E309A	5.4	4.4	18
K328A	3.8	3.8	0
H333A	8.6	6.7	23

[a] The % myristic acid transferred in 10 min from C_{14}-ACP to seven KAS I proteins/μg of protein was determined. The effect of ACP on transfer was assessed by adding 0.17 μM ACP (10 pmol/assay) prior to addition of KAS I. For C163S and K328A, 0.51 μM ACP (30 pmol/assay) was also used, giving the same results as were also obtained when the assays were carried out at pH 9.0. The protein concentrations which were in the linear range varied from 3 to 8 μg/assay, depending on the activity of the mutant. All assays were performed in triplicate for which the mean is presented. The SD for KAS I was ±0.3.

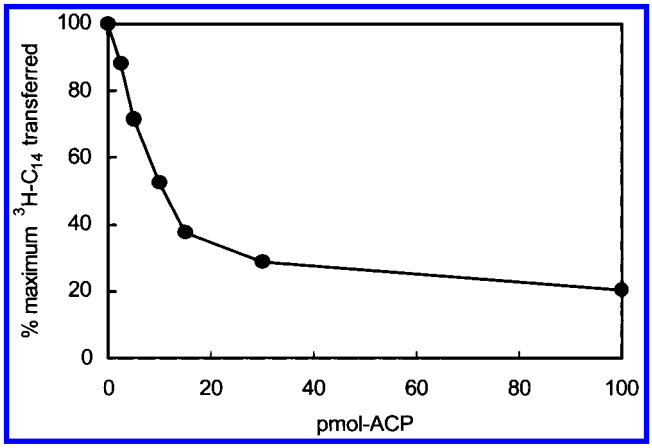

FIGURE 4: Inhibition of transfer of myristic acid from ACP to wild-type KAS I protein by ACP. KAS I protein was added to ACP (0−100 pmol, corresponding to 0−1.67 μM) and radiolabeled myristoyl-ACP in 50 mM K-PO_4, pH 6.8. The reaction mixture was incubated 10 min at 20 °C and injected onto the Superdex-200 size exclusion column. Fractions were collected for liquid scintillation as detailed under Materials and Methods. The % inhibition = % transfer (see Figure 3 caption) measured with ACP/% transfer when ACP was omitted from the incubation mixture.

that the transfer reaction was being inhibited. A likely inhibitory candidate is the ACP product of the reaction. The transfer of myristic acid to wild-type KAS I was therefore analyzed in the linear range in the presence of ACP. In these experiments, the acyl-ACP:KAS I subunit ratio is 1:4. ACP (0−100 pmol/assay) was added to the reaction mixture prior to initiating the reaction by addition of KAS I. As can be seen in Figure 4 ACP was an efficient inhibitor of KAS I. If one considers the sum of ACP + acyl-ACP:KAS I subunits, the ratio is approximately 1:1 unless KAS I exhibits negative cooperativity, in which case there will be an excess of ACP to KAS I. The physiological concentration of ACP, calculated from the data of Jackowski and Rock (*28*), is 0.14 μM in exponentially growing *E. coli* cells. A concentration of 0.14 μM corresponds to 8 pmol of ACP added per assay. We found that KAS I was about 50% inhibited at a concentration of 10 pmol per assay, indicating that KAS I is inhibited at the physiological concentration of ACP. Consequently, the mutants were analyzed at a concentration of 10 pmol of ACP per assay, corresponding to 0.17 μM (Table 2). Again the mutants could be divided into three groups: those that behaved as wild-type (H298A inhibited 45%), those where the percent inhibition was about half of wild-type (D306A, E309A, and H333A with 26, 18, and 23%, respectively), and finally those that were not inhibited (C163S and K328A). To analyze if the insensitivity of the latter two mutants was a result of a change in the pH optimum, the activity of the two proteins in the range from pH 5.0 to pH 9.0 was determined. K328A exhibited a weak optimum close to that of the wild-type whereas C163S seemed to have lost its optimum, being equally active from pH 5 to pH 9 (Figure 5). To confirm that the ACP inhibition was independent of pH, three transfer assays were performed for each of the mutants at pH 8.6 in the presence of 0, 10, and 30 pmol of ACP/assay. The acyl-ACP:KAS I subunit ratio was 1:4 in these experiments. Neither mutant was inhibited at the high pH at either ACP concentration (data not shown). As mentioned above, adding 10 pmol of ACP/assay (0.17 μM) after the reaction has gone to completion, and incubating 10 more min, had no effect on the amount of transfer, indicating that the reverse reaction under our conditions is negligible. The reverse reaction, however, has been observed when using 27 times excess of ACP to acyl-KAS I in the total absence of acyl-ACP (*29*).

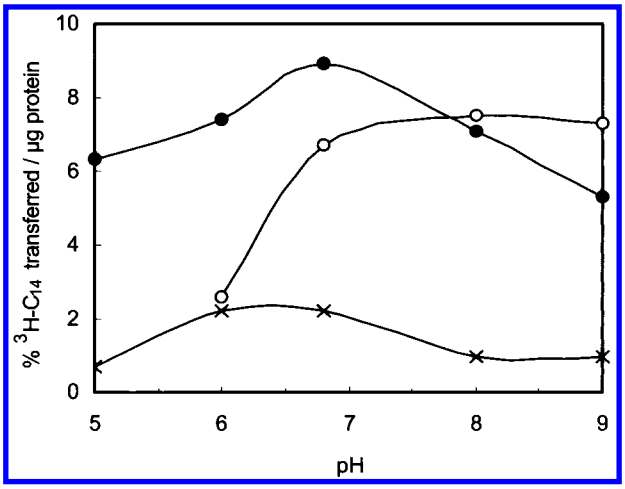

FIGURE 5: pH dependence of the transfer of myristic acid from ACP to wild-type, C163S, and K328A KAS I proteins. The 30 mM buffers used were citrate + K-PO_4 for pH 5−7, K-PO_4 for pH 6−8, and Tris-HCl for pH 8−9. ● = wild-type; ○ = C163S; × = K328A.

Decarboxylation of Malonyl-ACP by Wild-Type and Mutant KAS I Proteins. The ability of the KAS I proteins to decarboxylate the donor substrate malonyl-ACP to acetyl-ACP was determined by analyzing the trichloroacetic acid precipitable substrate and product on urea−13.3% PAGE. The amount of malonyl-ACP generated as substrate for the decarboxylation reaction is shown in the first lane of Figure 6A. Wild-type KAS I displays a constitutive level of acetyl-ACP formation that is dependent on the concentration of enzyme present as illustrated in lanes 2, 3, and 4. That less total labeled ACPs are visible in lane 3 suggests that derailment products such as triacetic acid lactone (TAL) (*30*) are being formed. The latter is not precipitable and hence will not be detected using our gel assay. TAL is synthesized when the C_2-unit arising from decarboxylation is transferred to the active site cysteine, where it acts as a primer substrate

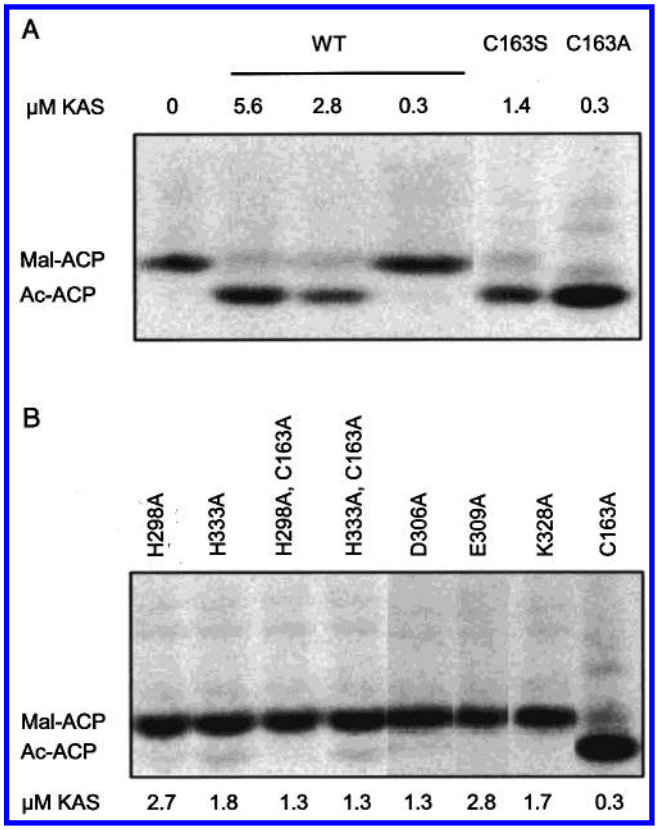

FIGURE 6: Ability of wild-type and mutant KAS I proteins to decarboxylate malonyl-ACP to acetyl-ACP. The μM KAS added per 100 μL assay is shown. Reaction was carried out as detailed under Materials and Methods for 10 min. After resolution of the ACP species by conformationally sensitive 13.3% acrylamide, 1 M urea gels, results were visualized by blotting to a PVDF membrane and autoradiography. WT = wild-type. (A) Wild-type and Cys-163 mutant KAS I proteins. (B) KAS I mutants severely impaired in acetyl-ACP synthesis compared to the very active C163A mutant.

for condensation. In the mammalian system, TAL and the C_4 intermediate β-ketobutryl-CoA account for 98% of the products recovered from a decarboxylation reaction with wild-type enzyme (*15*). We have detected neither a C_4- nor a C_6-ACP intermediate on our gels. The mammalian system clearly differs from *E. coli* KAS I in that acetyl-ACP can be a major product of the KAS I decarboxylation reaction in vitro (lane 2). TAL has been reported in *E. coli* (*31*, *32*). What derailment product(s) is (are) being formed in our assay is presently being investigated using *E. coli* KAS II protein, which in contrast to KAS I forms no detectable acetyl-ACP (*33*). Interestingly, while TAL is a derailment product of KAS enzymes, it is the natural product of the CHS-related pyrone synthase in *Gerbera hybrida* (*34*).

Both C163S and C163A mutant enzymes are capable of carrying out decarboxylation (lanes 5 and 6). Acetyl-ACP can be detected using as little as 0.01 μM C163A mutant enzyme/assay. That the intensity of the acetyl-ACP band observed when using the C163A protein can be greater than that of the starting substrate (lane 1 versus lane 6) presumably reflects the presence of the malonyl-ACP generating system combined with the fact that transfer of an acyl chain onto C163A is impossible, thereby prohibiting TAL synthesis. Regardless of the nature of the non-acetyl-ACP products of *E. coli* KAS I decarboxylation, our assay monitoring formation of acetyl-ACP and/or the disappearance of the malonyl-ACP substrate reveals whether a particular residue participates in the decarboxylation reaction.

The other five mutant proteins, H298A, D306A, E309A, K328A, and H333A, are severely impaired in decarboxylation at the tested protein concentrations (Figure 6B, lanes 1, 2, and 5−7). Only in the case of E309A is there any indication of a decrease in substrate (Figure 6B, lane 6). A subsequent experiment confirmed this, and revealed a clear decrease in substrate when 5.7 μM enzyme was used. Rather than increasing protein concentrations of the other KAS mutants to probe for traces of activity and to ascertain if the faint shadows, for example, in lanes 2 and 4, resulted from decarboxylation, we constructed double mutants of the five just mentioned with C163A which is unable to synthesize TAL. In no case was label readily detected in the position expected for the acetyl-ACP product as illustrated by the H298A and H333A double mutants (lanes 3 and 4). The three other double mutants were tested at the same protein concentration. These results suggest that the active site cysteine residue is not directly needed or involved in decarboxylation activity, in contrast to the other mutagenized residues which all produce enzymes defective in decarboxylation. We deduce, therefore, that the latter all contribute either functionally and/or structurally to the decarboxylation domain.

Elongation of Malonyl-ACP by Wild-Type and Mutant KAS I Proteins. The ability of the various KAS proteins to carry out the condensation reaction was assayed by testing whether they could restore elongation activity to a cerulenin-inhibited protein extract (CIPE). Figure 7A, lane 1, shows that in the absence of a KAS protein only the labeled donor substrate, malonyl-ACP, and several of the initial elongation products are readily detectable. The latter as well as longer acyl-ACPs are due to the presence of the cerulenin-nonsensitive KAS III in the protein extracts. The total amount of these acyl-ACPs varies from one CIPE preparation to another (compare Figure 7A,B, lanes 1). Despite this background difference, addition of wild-type KAS I (Figure 7A, lanes 2 and 9; Figure 7B, lane 2) results in excellent elongation activity. By comparison, addition of five mutant KAS proteins (Figure 7A, lanes 4−8) does not result in any increase in elongation ability beyond the CIPE background. The mutant D70A (lane 3) is just as active as the wild-type, however. This mutant was included as a random example of one of the highly conserved residues not predicted to participate in catalysis (*3*). The mutant KAS proteins C163S and E309A (Figure 7B, lanes 4 and 5) retain some elongation activity, but it is minor compared to that exhibited by the wild-type (lane 2). Using a quantitative assay, the mammalian analogue of the C163S mutation yielded a fatty acid synthase that had 5% of wild-type condensation activity (*37*). The analogous mutant in *E. coli* KAS III displayed a trace of activity (*7*), but that in *C. wrightii* exhibited none (*17*), as was also observed for that in CHS (*9*).

DISCUSSION

Independence of Decarboxylation and Transfer. A model of KAS catalysis proposed in 1993 predicted that two basic conserved residues facilitate decarboxylation (*3*): one by interacting with the thioester oxo group and the other with the oxo group of malonyl-ACP. The results of mutating His-

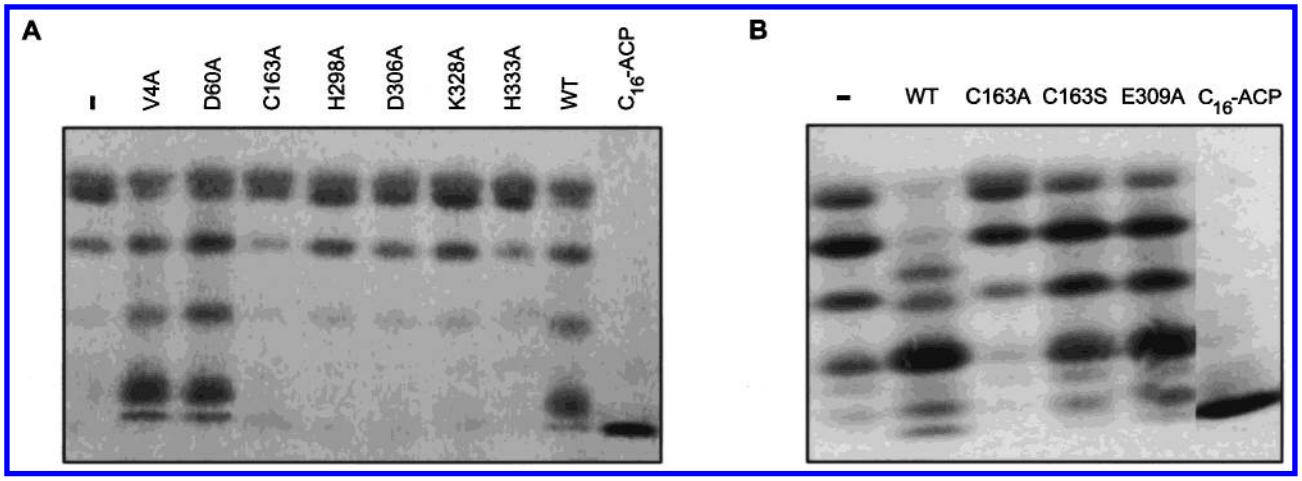

FIGURE 7: Ability of wild-type and mutant KAS I proteins to rescue cerulenin-inhibited protein extracts (CIPE). Reaction was carried out as described under Materials and Methods for 20 min at 37 °C with 0.2−0.4 μg of KAS protein/assay. After resolution of the ACP species by conformationally sensitive 13.3% acrylamide, 4 M urea gels, results were visualized by blotting onto a PVDF membrane and autoradiography. (A and B) Activities of CIPE preparations when no KAS protein (−), or wild-type (WT) and mutant KAS proteins are added. [^{14}C]-C_{16}-ACP standard. V4A, which exhibits wild-type activity, is the original mutant KAS from which the WT and other mutants have been derived (see Materials and Methods).

298 and His-333, combined with structural analyses and modeling of malonyl-ACP into KAS I, have now lead to precise roles for two histidines (*10*). Briefly, His-298 acts as a general base and abstracts a proton from the carboxylic leaving group of malonyl-ACP. His-333 donates a hydrogen bond to the oxo group of malonyl-ACP. The coordinated action of both histidines is required for decarboxylation. That both the C163S and C163A mutant proteins are efficient decarboxylators implies that the active site cysteine is not involved in the decarboxylation reaction. This is in agreement with the results obtained from analyses of the transfer reactions. The same conclusion was drawn for the mutant analogues of CHS exhibiting only 10% of wild-type activity (*9*), and for the serine mutant of KAS III from *E. coli* which manifested a 425% increase in activity compared to wild-type (*7*). By comparison, mutating KAS III from *C. wrightii* to the C163A analogue abrogated detectable activity although the C163S analogue exhibited good activity (*17*).

Another function attributed to a conserved basic residue in earlier models of catalysis was that of a general base abstracting a proton from the active site cysteine, enabling it to act as a nucleophile and accept a primer substrate for elongation (*3*, *5*). Mutating either His-298 or His-333 to alanine affected transfer of C_{14} acyl chains to the active site cysteine of KAS I differently. The activity observed when a mutation is present compared to that of the wild-type reveals the contribution of the nonmutated residues to the transfer reaction. The 35% increase in transfer efficiency characterizing H333A thus infers that a histidine at position 298 is sufficient for transfer, while the 34% decrease in transfer efficiency exhibited by H298A reveals that a single histidine at position 333 is insufficient for full transfer activity. These modest changes are unlikely to impair transfer and are similar to the observation that mutating the active site histidine and aspargine to alanines in *E. coli* KAS III does not impede transfer activity (*7*). The same is true for the histidine mutant of CHS although mutation of the asparagine to alanine resulted in a significant reduction in transfer activity (*9*). Our results do not support the model referred to above in which one of the specified residues acts as a general base. They are, however, in accord with our deductions from structural analyses (*10*) revealing that the nucleophilicity of the active site cysteine is a result of two other factors. First, Cys-163 is positioned at the N-terminus of an α-helix, allowing it to exploit the α-helix dipole moment, thereby becoming nucleophilic. Second, the oxo group of the bound fatty acid is positioned in an oxyanion hole defined by the backbone amide groups of Phe-392 and Cys-163 that increases the electrophilic nature of the C_1 acyl carbon, thus assisting its attack on the cysteine. The same model has also been deduced for *E. coli* KAS III (*7*) and CHS (*9*). That is, neither the active site histidine nor the asparagine functions as a general base for abstracting a proton from the active site cysteine. That recent analyses of *C. wrightii* KAS III mutants support the original contention, identifying the His-298 analogue as the basic residue (*17*), is puzzling. Structural analyses of this enzyme should throw light on this unexpected difference. By contrast, the TLS reaction is thought to be initiated by the histidine analogue of His-333 abstracting a proton from the active site cysteine (*12*, *36*).

Asp-306 and Glu-309 Are Required for Decarboxylation but Not for Transfer. Roles in maintenance of the active site scaffold have been assigned to two acidic residues in KAS II which are equivalent to Asp-306 and Glu-309 in KAS I (*5*). The same deduction from structural considerations can be made for the KAS I residues (*4*). The significance of the proposed backbone stabilization to catalysis was revealed by mutating these acidic residues to alanine. While decarboxylation activity, and hence condensation activity, was not exhibited by D306A mutant protein, traces of both activities were detected with that of the E309A mutant protein. This implies that backbone stabilization associated with Asp-306 is somewhat more important for decarboxylation than that contributed by Glu-309. This was not predicted as Glu-309 bonds directly to Gly-299 and His-298 which is essential for decarboxylation, while Asp-306 bonds directly to Ser-301 and via a water molecule to Thr-300 at the base of the entrance channel (Figure 8). Thr-300, however, is as Thr-302 within hydrogen bonding distance of the oxo group of the CO_2 that is released during decarboxylation, as deduced from modeling studies (Figure 7 in *10*). With respect to transfer activity, both mutations resulted in only modest

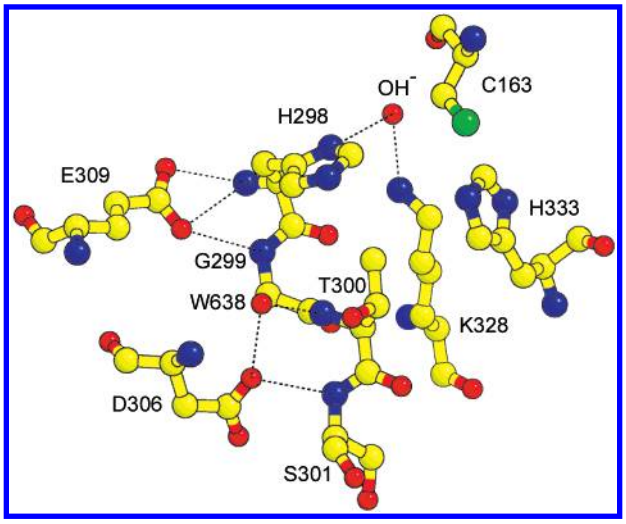

FIGURE 8: Mutation of Asp-306, Glu-309, and Lys-328 to alanines results in KAS I enzymes that are severely impaired in decarboxylation. The hydrogen bonds that would be lost by these mutations are shown as dotted lines. Most important are (i) those from Glu-309 to the backbone of His-298, one of the two catalytic residues required for decarboxylation; (ii) that from Asp-306 to water 638 (W638), which in turn can hydrogen-bond to the backbone of Thr-300 that modeling (*11*) position within hydrogen bond distance of the leaving carbonyl of malonyl-ACP; and (iii) that from Lys-328 to a hydroxide ion (OH^-), which is part of the hydrogen bond network predicted (*11*) to enable His-298 to abstract a proton from the carboxylic leaving group of malonyl-ACP. Yellow, carbon; red, oxygen; blue, nitrogen; green, sulfur. Figure prepared using Molscript (*47*) and Bobscript (*48*).

alterations in activity, revealing that backbone stabilization by these two residues is insignificant for this reaction.

Functions for Lys-328. The most enigmatic of the completely conserved residues in the active site is Lys-328. It is located quite distant from the active site cysteine at the base of the active site pocket with its hydrophobic arm ending in a plus charge extending upward toward the two histidines as illustrated in Figure 8 (*4*). When mutated to alanine, a very interesting KAS enzyme is produced which is severely impaired in decarboxylation and hence condensation, but also exhibits very unusual kinetics for the transfer reaction. While transfer efficiency is significantly slowed revealing apparent sigmoidal kinetics, 100% of the myristic acid is eventually transferred to the mutant K328A. In mammalian FAS, mutation of the analogous lysine to alanine also results in an enzyme impaired in condensation activity (*35*, *37*). Based on the distance from the active site cysteine and participation in a salt bridge to a nearby glutamic acid residue, a strictly structural role for the lysine was proposed (*5*). Our crystallographic analyses, however, have revealed that Lys-328 is connected to His-298 by a hydroxide ion (see Figure 8) that is part of a hydrogen bond network. The latter is proposed to destabilize His-298, enabling it to act as a general base to initiate the decarboxylation reaction (*10*). If this is the only function of Lys-328, then the observed slowing down of transfer of the acyl substrate to the mutant K328A enzyme is not readily explicable. Based on its hydrocarbon chain and charge, we suggest that Lys-328 must have an additional function. The defect in transfer infers a very early role in catalysis, for example, in assisting the fatty acid in entering the enzyme and arriving in the substrate binding pocket. This would involve an interaction between KAS residues and the fatty acid chain. Such an interaction would be promoted via carbon-rich side chains and/or electrostatic interactions, both characteristics of lysine. Such a role has been presented for Arg-56 in the intestinal fatty acid binding protein (IFABP) (*38*). Arg-56 is, as Lys-328, buried in the protein in the crystal structure making several hydrogen bonds to adjacent residues, although the NMR structure of IFABP shows that this arginine in the soluble protein points outward from the surface, and lacks both hydrogen bonds and salt bridges. Arg-56 is thus able to interact with the fatty acid through electrostatic interactions (*39*). In this protein family, this residue is always an arginine or lysine. Both these amino acids in apolipoprotein AI have been found to interact with phospholipids in high-density lipoproteins. This interaction is accomplished through the hydrocarbon-rich side chains (*40*). As an initial step in testing these proposals, we are presently examining the effect on the KAS I transfer reaction of mutating Lys-328 to appropriate amino acids.

ACP Released during Transfer Inhibits KAS I. In an exponentially growing *E. coli* cell, ACP is one of the most abundant soluble proteins with an estimated number of 6×10^4 molecules per cell (*41*). All the ACP is believed to bear 4′-phosphopantetheine as no significant pool of ACP lacking this prosthetic group was detected (*28*). The present experiments reveal that under physiological conditions ACP generated during the transfer of an acyl substrate to KAS I decreased transfer of the acyl substrate to KAS I by ca. 50%. The reverse reaction did not take place, as addition of ACP after the transfer reaction had gone to completion had no effect on transfer efficiency. This inhibition must leave the enzyme sufficiently active to synthesize the requisite amounts of fatty acids. Given that this simulates the in vivo situation, such a regulatory mechanism would allow a very sensitive way to adjust the number of fatty acid chains being elongated. Both KAS II and KAS III are insensitive to ACP (*27*, *42*, *43*). By comparison, long-chain acyl-ACPs feedback-inhibit KAS III (*42*−*44*).

While the same level of inhibition of transfer was characteristic for the mutant H298A enzyme, the others investigated were less compromised, namely, D306A, E309A, and H333A by ca. half as much, C163S and K328A not at all. This infers that mutation of these buried residues alters the surface potential so that ACP recognition is affected. The highly positive region on the surface of KAS I encompassing the active site entrance has been suggested as the docking site for the highly negative ACP when it delivers its acyl chain to the enzyme (*4*). Very recently the malonyl-ACP docking site on KAS III was assigned to an analogous highly positively charged region adjacent to the active site entrance by predicting the residues participating in the ACP−KAS III interaction and analyzing the effect of mutating them. The crucial residue was Arg-249 (*45*). Mutational analyses of *C. wrightii* KAS III identified several residues which upon mutation yielded enzymes that were insensitive to acyl-ACPs while still retaining most of their catalytic activity (*46*). In KAS III from *E. coli*, all three residues are buried beneath the positively charged region containing the ACP docking site (*45*). These mutations were presumed to affect the surface potential of the docking site for the acyl-ACPs involved in feedback regulation (*46*). That buried residues upon mutation are able to alter surface characteristics is in accord with the suggestion presented above for the newly proposed function of Lys-328. Whether a single docking site

for the malonyl- and acyl-ACP substrates as well as ACP inhibition will suffice for KAS I, or if more than one site is required is presently unknown.

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