Mechanism of the β-Ketoacyl Synthase Reaction Catalyzed by the Animal Fatty Acid Synthase†

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ABSTRACT: The catalytic mechanism of the β-ketoacyl synthase domain of the multifunctional fatty acid synthase has been investigated by a combination of mutagenesis, active-site titration, product analysis, and product inhibition. Neither the reactivity of the active-site Cys161 residue toward iodoacetamide nor the rate of unidirectional transfer of acyl moieties to Cys161 was significantly decreased by replacement of any of the conserved residues, His293, His331, or Lys326, with Ala. Decarboxylation of malonyl moieties in the fully-active Cys161Gln background generated equimolar amounts of acetyl-CoA and bicarbonate, rather than carbon dioxide, and was seriously compromised by replacement of any of the conserved basic residues. The ability of bicarbonate to inhibit decarboxylation of malonyl moieties in the Cys161Gln background was significantly reduced by replacement of His293 but less so by replacement of His331. The data are consistent with a reaction mechanism, in which the initial primer transfer reaction is promoted largely through a lowering of the pKa of the Cys161 thiol by a helix dipole effect and activation of the substrate thioester carbon atom by binding of the keto group in an oxyanion hole. The data also indicate that an activated water molecule is present at the active site that is required either for the rapid hydration of carbon dioxide, prior its release as bicarbonate or, alternatively, for an initial attack on the malonyl C3. In the alternative mechanism, a negatively-charged tetrahedral transition state could be generated, stabilized in part by interaction of His293 with the negatively charged oxygen at C3 and interaction of His331 with the negatively charged thioester carbonyl oxygen, that breaks down to generate bicarbonate directly. Finally, the carbanion at C2, attacks the electrophilic C1 of the primer, generating a second tetrahedral transition state, also stabilized through contacts with the oxyanion hole and His331, that breaks down to form the β-ketoacyl-S-acyl carrier protein product.

The β-ketoacyl synthases catalyze the formation of new carbon−carbon bonds by condensation of a variety of acyl-chain precursors with an elongating carbon source, usually malonyl or methylmalonyl moieties, that are covalently attached in thioester linkage to an acyl carrier protein (ACP).[1] These enzymes exist in two different molecular forms. In plants and the majority of prokaryotes, the enzymes consist of approximately 400 residues and typically form homodimers: the so-called monofunctional polypeptide, or type II systems (*1*, *2*). On the other hand, in the fatty acid synthesizing systems of animals and the structurally related modular polyketide systems of prokaryotes, the β-ketoacyl synthases constitute one of the catalytic domains of large multifunctional polypeptides, the type I FASs and polyketide synthases (*3*−*5*). In the type II FAS systems of plants and microorganisms, multiple forms of β-ketoacyl synthases have been described that have different substrate specificities. β-Ketoacyl synthase III uniquely catalyzes the elongation of an acetyl-CoA primer by malonyl-ACP, whereas β-ketoacyl synthases I and II, which have overlapping specificities, utilize only acyl-ACPs for elongation by malonyl-ACP (*2*, *6*−*8*). Both β-ketoacyl synthases I and II can effectively elongate short chain-length precursors to the 14-carbon stage, but further elongation, in particular the elongation of C16:1-ACP to C18:1-ACP, is more efficiently catalyzed by β-ketoacyl synthases II. A type II β-ketoacyl synthase in *Mycobacterium tuberculosis* specializes in elongating acyl-ACPs containing more than 26 carbon atoms and plays an important role in the production of mycolic acids by this organism (*9*). In the animal type I FAS, a single β-ketoacyl synthase catalyzes all of the condensation reactions necessary for elongation of a two-carbon precursor to palmitic acid; this enzyme has very limited ability to elongate the 16-carbon acyl chain (*10*−*12*), which is consequently removed from the ACP domain through the action of a chain-terminating thioesterase (*13*−*16*). Thus, the specificities of the various types of β-ketoacyl synthases that have been characterized clearly play an important role in determining the nature of the products formed in the respective biosynthetic pathways.

The overall reaction catalyzed by the β-ketoacyl synthase can be divided into three discrete steps: [i] transfer of the acyl moiety from its acyl carrier protein thioester form (or acyl-CoA, in the case of β-ketoacyl synthase III) to the active-site cysteine residue of the β-ketoacyl synthase, [ii] binding and decarboxylation of the chain extender moiety to yield the a reactive carbanion, and [iii] formation of a

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[1] Abbreviations: FAS, fatty acid synthase; ACP, acyl carrier protein; hplc, high-performance liquid chromatography.

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	163		298		328	333		392	
I, *E. coli*	SA**C**ATS		NS**H**GTST		AT**K**AMTG**H**SL			NSFG**F**GG	
	163		303		335	340		400	
II, *E. coli*	TA**C**TSG		NA**H**GTST		ST**K**SMTG**H**LL			NSFG**F**GG	
	167		307		339	344		403	
II, *Synechocystis*	TA**C**TSG		NA**H**GTST		ST**K**SMTG**H**LL			NSFG**F**GG	
	161		293		326	331		395	
Animal	TA**C**SSS		EA**H**GTGT		ST**K**SNMG**H**PE			NSFG**F**GG	
	112		244			274		306	
III, *E. coli*	AAC**A**GF		VP**H**QANL			G**N**TS		EAFG**G**GF	

FIGURE 1: Location of catalytically important residues in β-ketoacyl synthases. The sequences for the β-ketoacyl synthase domains of human, rat, and chicken FASs are identical in the regions shown.

new carbon−carbon bond by nucleophilic attack of the carbanion on the carbonyl carbon of the acyl moiety (*17*). Multiple sequence alignments clearly indicate that the β-ketoacyl synthase domain of the animal FAS and its type II counterparts share a similar primary structure and most likely, therefore, a similar reaction mechanism (*17*). In addition to the cysteine nucleophile, two histidines and a lysine residue are conserved in all but the β-ketoacyl synthase III forms of the enzyme and are believed to play essential catalytic or structural roles (Figure 1).

Crystal structures of all three types of *Escherichia coli* β-ketoacyl synthases, I (*18*), II (*19*), and III (*20*−*22*) and the β-ketoacyl synthase II from the cyanobacterium *Synechocystis sp.* (*23*) have been determined. All are dimers exhibiting an α-β-α-β-α fold and their active centers are characterized by the presence of a Cys-His-His triad, in β-ketoacyl synthases I and II, and a Cys-His-Asn triad in β-ketoacyl synthase III (Figure 1). Surprisingly however, these three-dimensional structures have yet to be translated into a clear consensus as to the roles played by the universally conserved amino acid residues in this class of condensing enzymes. In this study, we have utilized a combination of mutagenesis, active-site titration, pH/activity, and inhibitor studies, in an attempt to elucidate details of the catalytic mechanism employed by the β-ketoacyl synthase domain in the context of the animal FAS complex.

EXPERIMENTAL PROCEDURES

Materials. All radioactive reagents were obtained from Moravek Biochemicals (Brea, CA). Synthesis of [1-^{14}C]-decanoyl-CoA was described previously (*12*). Other reagents were purchased from Sigma (St. Louis, Mo) unless otherwise indicated.

Construction of cDNAs Encoding 6xHis- and FLAG-Tagged FASs and Expression of the Proteins in Sf9 cells. The strategy for construction of cDNAs encoding the wild-type FAS, single domain specific mutants and incorporation of a 6xHis or FLAG tag has been described in detail elsewhere (*24*−*27*). Constructs encoding the β-ketoacyl synthase domain mutants His293Ala and His331Ala were engineered by first generating mutated cDNA fragments, by the overlap polymerase chain reaction, using pFAS 74.20 (partial FAS cDNA in pUCBM20) as template (*28*). Primer sets[2] M293T/54B1152, 27T330/M293B (for His293Ala) and M331T/54B1152, 27T330/M331B (for His331Ala) were used for the first round of polymerase chain reactions, whereas the second rounds of amplification were carried out using the 27T330/54B1152 set of primers. Authenticity of the amplification product was confirmed by DNA sequencing, then, using available unique restriction sites, the DNA was moved stepwise into the full-length wild-type construct pFAS 219.FB, which also encodes a carboxyterminal 6xHis tag.

The various double, triple, and quadruple mutants were assembled stepwise by incorporating cDNA fragments carrying individual mutations into the full-length FAS cDNA. At each step, authenticity of the new substitution was confirmed by DNA sequencing. The final FAS cDNA constructs, in the context of the pFASTBAC 1 vector (FB), were used to generate recombinant baculovirus stocks by the transposition method, employing the BAC-to-BAC baculovirus expression system according to the manufacturers instructions. *Sf*9 cells were then infected with the purified recombinant viruses and cultured for 48 h at 27 °C. The tagged FAS proteins were purified from the cytosols as described earlier (*24*); glycerol (10%, v/v) was included in all buffers used for chromatography.

Synthesis of Decanoyl Thioesters. Synthesis and purification of S-decanoyl pantetheine and S-decanoyl-*N*-acetylcysteamine was described previously (*12*, *15*). S-decanoyl *N*-(*N*-acetyl-β-alanyl)-cysteamine was synthesized as follows: 1 mmol each of *N*-acetyl-β-alanine (Novabiochem, Darmstadt, Germany), *N*-hydroxysuccinimide, and *N*,*N*′-dicyclohexylcarbodiimide (Novabiochem) were stirred in 20 mL tetrahydrofuran:ethyl acetate (3:2) for 3 h at 20 °C. Precipitated dicyclohexylurea was separated by filtration, the solvent was evaporated, and the resulting oil was dissolved in 8 mL tetrahydrofuran. Cystamine hydrochloride, 0.5 mmol in 0.2 M sodium carbonate, was added, the pH was adjusted to 7.5−8 with sodium hydroxide, and the reaction mix was left stirring overnight at 20 °C. The remaining cystamine was acetylated with an excess of acetic anhydride. Complete acetylation was verified by silica thin-layer chromatography using dichloromethane:methanol (18.5:1.5) as solvent, followed by detection with ninhydrin. The solution was acidified and solvent evaporated. The oily precipitate was dissolved in 8 mL tetrahydrofuran/1 mL water and the disulfide reduced by stirring with 1.2 mmol of tributylphosphine (Aldrich) for 15 min. The pH of the mixture was adjusted to 8 by addition of 1 mL of 2 M sodium carbonate. A slight excess of decanoyl chloride was added and the pH maintained at ∼8 by addition of a solution of sodium hydroxide. When the reaction was completed, the pH was adjusted to ∼2. The upper layer was extracted twice with 10 mL of petroleum ether and the extract left in the refrigerator overnight. The precipitate formed was separated and recrystallized twice from 25 mL water at 60 °C, dissolved in 5 mL methylene chloride to remove water (upper layer), and, after addition of 20 mL petroleum ether, recrystallized at 4 °C. The monoisotopic molecular mass of the product, determined by electrospray mass spectrometric analysis, was 343.1 Da, close to the expected value of 343.5 Da. The purity of the product was estimated by thin-layer chromatography in dichloromethane:methanol (18.5:1.5, R_f = 0.42) to be

[2] Primer sequences: M293T 5′-AATATATTGAAGCCRCAGGCACGGGCACCAA; M293 5′-TTGGTGCCCGTGCCTGYGGCTTCAATATATT; M331T 5′-AAATCCAACATGGGARCACCTGAGCCTGCCT; M331B 5′-AGGCAGGCTCAGGTGYTCCCATGTTGGATTT; 27T330 5′-GTC AGC TAT GAA GCT ATT GTG; 54B1152 5′-CACTAGAATTCTTCAGGGTTGGGGTTGTGGAAATGC.

~90%. The major contaminant was S-decanoyl-*N*-acetylcysteamine.

Assay of Overall Fatty Acid Synthesizing Activity. The activity was measured spectrophotometrically at 37 °C (*29*).

Assay of β-Ketoacyl Synthase Activity. The activity was assayed by chromatographic detection of the radiolabeled β-ketobutyryl-CoA formed from [2-^{14}C]malonyl-CoA and acetyl-CoA in the absence of NADPH (*25*). CoASH was freshly prepared or used from a stock of CoASH containing an equimolar amount of tris(2-carboxyethyl)phosphine (Calbiochem-Novabiochem Corp., San Diego, CA); both preparations gave identical results. A FAS mutant (S2151A) lacking the phosphopantetheine moiety in the ACP domain was used as a control to estimate nonspecific background activity.

Assay of Malonyl Decarboxylase Activity. Decarboxylase activity was assayed by quantification of radioactive acetyl-CoA, β-ketobutyryl-CoA, and triacetic acid lactone formed from [2-^{14}C]malonyl-CoA in the presence of CoASH, after separation of the reaction components by hplc (*30*).

Carbon Dioxide State Assay. The rate of bicarbonate formation during malonyl decarboxylation by C161Q FAS was measured in a coupled assay developed originally in the laboratory of Frank Jordan (Eduard Sergienko, personal communication, *31*). Reaction mixtures contained 100 mM tris-HCl (pH 7), 10 mM $MgCl_2$, 0.4 mM NADH, 4 mM phospho(enol)pyruvate, 50 μM CoASH, 100 U/ml pig heart malate dehydrogenase (Roche Molecular Biochemicals, Indianapolis, IN), and 2 U/ml maize leaf phospho(enol)-pyruvate carboxylase (Fluka Chemical Corp., Milwaukee, WI). The reaction mix, containing all components except FAS and malonyl-CoA, was divided into two approximately equal volumes. FAS, 0.1 mg/mL final concentration, was added to one portion and malonyl-CoA, 110 μM final, to the second portion, and mixtures were separately preincubated for 2 min to remove residual bicarbonate. The two portions were mixed together, and NADH oxidation was recorded at 340 nm. When the G1888A FAS mutant was assayed, the reaction was supplemented additionally with 53 μM acetyl-CoA to allow the condensation reaction to occur. For experiments using pyruvate decarboxylase in place of FAS, 15.5 mU/ml yeast pyruvate decarboxylase was assayed under similar conditions, with the exception that the reaction was supplemented with 1 mM thiamine pyrophosphate and 22 mM sodium pyruvate, and both FAS substrates were omitted. The total rate of formation of both bicarbonate and carbon dioxide was assayed by supplementing the reaction mixture with 500 U/ml of bovine erythrocyte carbonic anhydrase. All reactions were carried out at 25 °C. To minimize the background level of carbon dioxide present, nitrogen was bubbled through all solutions, with the exception of enzymes and malonyl-CoA, and optical cells were presoaked in 0.1 M hydrochloric acid.

Unidirectional Acyltransferase Reaction. FAS preparations, at 4.6 μM, were incubated with 0.56 mM [1-^{14}C]-butyryl-CoA at room temperature, and after 1, 2, 5, and 8 min, portions of the reaction mixture were transferred to perchloric acid (final concentration 10%). The precipitates were separated by centrifugation, washed four times with 5% trichloroacetic acid, and dissolved in 6 M guanidine hydrochloride, and radioactivity was determined by liquid scintillation spectrometry.

Bidirectional Acyltransferase Reaction. A model substrate assay was employed involving transesterification of decanoyl moieties from pantetheine, *N*-acetylcysteamine, or *N*-acetyl-N-β-alanyl-cysteamine thiol to CoASH thiol and separation of substrates and product by hplc (*12*).

Modification and Inactivation by Iodoacetamide. The FAS storage buffer was replaced with 0.2 M potassium phosphate, pH 6.3, containing 1 mM EDTA, 10% glycerol, and 0.5 mM tris(2-carboxyethyl)phosphine, by dialysis in a mini Slide-A-Lyzer 7,000 (Pierce, Rockford, IL) for 3−4 h at room temperature. The modification reaction was carried out at 37 °C for various time intervals and the reaction quenched by addition of trichloroacetic acid (10%, final concentration). Precipitates were separated by centrifugation, washed five times with 5% trichloroacetic acid, and solubilized in 6 M guanidine hydrochloride. Radioactivity was assayed by liquid scintillation spectrometry. A quadruple mutant (Ser581Ala, Ser2151Ala, Ser2302Ala, Cys161Ala) was used as a control to determine nonspecific modification.

Nonradioactive iodoacetamide was used for inactivation of FAS. At various time intervals, portions of the iodoacetamide-treated FAS were transferred directly to tubes containing the decanoyl transferase assay cocktail and 1 mM dithiothreitol. For each FAS mutant, control experiments were performed to estimate the effect on activity of the small amount of iodoacetamide transferred to the assay system along with the treated protein. At zero time, iodoacetamide, even at the highest concentration used to treat the FAS (0.23 mM), caused less than 10% inhibition in the acyltransferase assay.

RESULTS

Reactivity of the Active-site Cysteine and Transfer of the Acyl Substrate. The location of the active-site cysteine residue in the β-ketoacyl synthase domain of the rat FAS was previously identified as Cys161 (*25*, *32*). In preparation for the condensation step, the growing fatty acyl chain is transferred from the ACP domain phosphopantetheinyl (at Ser2151) to the Cys161 thiol, in a reaction catalyzed by the β-ketoacyl synthase domain (*12*). To evaluate the possible role of the conserved basic residues in activation of the Cys161 nucleophile, we utilized iodoacetamide as a probe. Earlier studies in other laboratories demonstrated that, at pH ~6, iodoacetamide modifies the active-site cysteine of the animal FAS with very high selectivity (*33*−*36*). Since iodoacetamide reacts with the thiolate anion of cysteine, the rate of alkylation provides a convenient measure of the ionization state of the sulfur atom (*37*). In these experiments, we evaluated the effect on the cysteine ionization state of replacing each of the conserved basic residues in the context of a triply mutated FAS, Ser581Ala, Ser2151Ala, Ser2302Ala, in which the nucleophilic residues in the malonyl/acetyl transferase, ACP, and thioesterase domains, respectively, had been replaced with alanine. In a control experiment, we utilized a quadruple mutant, Cys161Ala, Ser581Ala, Ser2151Ala, Ser2302Ala, to assess the extent of "nonspecific" reaction of iodoacetamide with thiols other than Cys161. At pH 6.3, the specific reaction of iodoacetamide at 0.23 mM with the Cys161 thiol of the triply mutated FAS accounted for 40% of the total thiols reacted (FAS contains ~40 surface accessible cysteine residues per subunit, *38*). Exploration of the effect of modification of Cys161 by iodoacetamide at

Table 1: Effect of Replacement of the Conserved Basic Residues on the Reaction of the Cysteine Nucleophile with Iodoacetamide

	FAS mutant			
	triple mutant[a]	quadruple mutant (His293Ala)	quadruple mutant (His331Ala)	quadruple mutant (Lys326Ala)
iodoacetamide modification[b]				
$k_{modification}$ ($s^{-1}mM^{-1}$)	0.014	0.281	0.276	0.023
$k_{relative}$ (ratio)	1	20	19	1.6
inactivation by iodoacetamide[c]				
$k_{inactivation}$ ($s^{-1}mM^{-1}$)	0.0028	0.078	0.080	n.d.
$k_{relative}$ (ratio)	1	28	29	

[a] Triple mutant: Ser581Ala, Ser2151Ala, Ser2302Ala. Quadruple mutants contains three mutations of the triple mutant and an additional mutation indicated in the parenthesis. [b] Nonspecific binding of iodoacetamide, assessed using FAS containing the triple mutation plus the Cys161Ala mutation, was ~8% of the total binding at 90 μM iodoacetamide (used for FASs containing the histidine mutations) and ~60% at 230 μM iodoacetamide (used for FASs containing the Lys326Ala mutation or the triple mutations alone); the control values for nonspecific binding have been subtracted from the experimental values to derive the data shown. Rate constant were calculated using the Guggenheim method with Δt equal to 30 s (*39*). [c] Inactivation was monitored using the bi-directional acyltransfer reaction and data were calculated from the plot ln(% activity) = *f*(time). n.d., not determined.

higher pH was hampered by a substantial increase in this nonspecific reaction of iodoacetamide with the FAS. Unexpectedly, we found that replacement of either His293 or His331 with alanine actually increased the rate of Cys161 carboxyamidomethylation; values of the second-order rate constants rose more than 20-fold (Figure 2A and Table 1, top section). Replacement of Lys326 had no effect on the rate of carboxyamidomethylation. Clearly, reactivity of the Cys161 thiol is not dependent on the presence of any of the conserved basic residues.

Two different assay systems, unidirectional and bidirectional, were employed to assess the acyltransferase activity associated with the β-ketoacyl synthase domain, again in the context of the triply mutated Ser581Ala, Ser2151Ala, Ser2302Ala FAS. In the unidirectional assay, where the ability of the β-ketoacyl synthase domain to transfer an acyl moiety from CoA thioester to the Cys161 nucleophile was measured directly, the rate of transfer of acyl moieties to the Cys161 thiol was not seriously compromised by replacement of any of the basic residues.

Activity was actually increased severalfold in the His293Ala mutant and reduced only by ~50% in the Lys326Ala mutant (Table 2). At saturation, only about half of the Cys161 residues were occupied by butyryl moieties (Table 2). Similar results were obtained for the triple mutant using [1-^{14}C]-decanoyl-CoA as acyl donor (data not shown).

In the bidirectional assay system, where a model thiol acceptor was included and the rate of acyltransfer from donor to acceptor thiol was assessed, again the rate of acyltransfer by the His293Ala mutant was elevated significantly compared to that of the triply mutated control. However, the activity of the His331Ala mutant was moderately reduced, and the activity of the Lys326Ala mutant was almost completely eliminated (Table 3). The apparent involvement of Lys326 in the bidirectional acyl transfer reaction but not in the unidirectional reaction suggested that perhaps, in the bidirectional assay, it is the access of the thiol acceptor substrate that is compromised by the Lys326Ala mutation. Indeed, in the Lys326Ala mutant, when decanoyl-CoA was used as acyl donor and pantetheine as acceptor, the K_m for pantetheine was increased approximately 36-fold, compared to that for the wild-type FAS, with only a slight reduction in the K_m for the donor substrate (data not shown).

Concurrent determination of the β-ketoacyl synthase acyltransferase activity (bi-directional assay) in the various

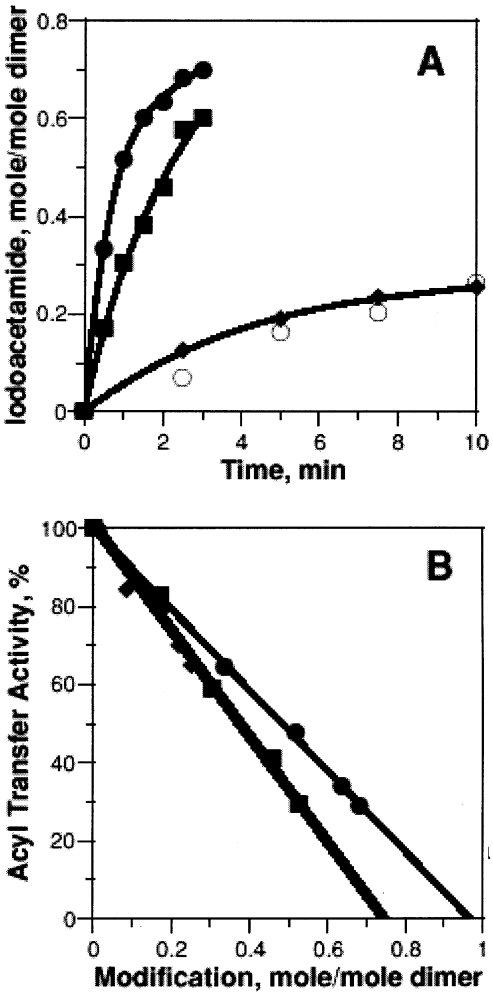


FIGURE 2: Modification and inactivation of FAS β-ketoacyl synthase mutants by iodoacetamide. The additional mutations introduced to give quadruply mutated FASs are denoted below. (A) The triple mutant (○) and the quadruple mutant containing the Lys326Ala mutation (◆) were incubated with 0.23 mM [1-^{14}C]-iodoacetamide for the indicated time. Quadruple mutants containing the His293Ala (●) and His331Ala (■) mutations were incubated with 0.09 mM ^{14}C-iodoacetamide. (B) Plot of inactivation vs modification.

FAS mutants treated with iodoacetamide (Figure 2B) revealed similar second-order rate constants for transferase inactivation and modification by iodoacetamide (Table 1, lower section). This finding was expected, based on the

Table 2: Unidirectional Acyltransferase Activity of FAS Mutants[a]

	FAS mutant			
	triple mutant[b]	quadruple mutant (His293Ala)	quadruple mutant (His331Ala)	quadruple mutant (Lys326Ala)
initial rate mmole/(mole dimer s)	18.4	80.4	14.0	7.6
saturation loading mole/mole dimer	0.98	0.82	0.92	0.92

[a] The substrate was butyryl-CoA. [b] Triple mutant: Ser581Ala, Ser2151Ala, Ser2302Ala. Quadruple mutants contains three mutations of the triple mutant and an additional mutation indicated in the parenthesis.

Table 3: Bidirectional Acyltransferase Activity of FAS Mutants[a]

	FAS mutant			
	triple mutant[b]	quadruple mutant (His293Ala)	quadruple mutant (His331Ala)	quadruple mutant (Lys326Ala)
k_{cat} (s^{-1})	1.14	3.47	0.29	0.02
K_m (mM)	0.11	0.04	0.05	0.05
k_{cat}/K_m (s^{-1} mM^{-1})	10.3	86.6	5.8	0.41

[a] The acyl donor substrate was decanoyl pantetheine and the acceptor was CoASH. [b] Triple mutant: Ser581Ala, Ser2151Ala, Ser2302Ala. Quadruple mutants contains three mutations of the triple mutant and an additional mutation indicated in the parenthesis.

assumption that iodoacetamide targets highly specifically the Cys161 nucleophile, as argued above. Fifty percent inhibition of the acyltransferase activity of the quadruple mutants containing the His293Ala and His331Ala mutations within the β-ketoacyl synthase domain was associated with the modification of only 0.4−0.5 mol thiol residues per mol FAS dimer and extrapolation of the inhibition/modification line suggests that complete inhibition likely may be achieved by modification of only one thiol residue per dimer (Figure 2B).

In the β-ketoacyl synthase-mediated acyl transferase assay, the Ser581Ala, Ser2151Ala, Ser2302Ala triple mutant exhibited significantly higher activity when pantetheine, rather than *N*-acetyl-N-β-alanyl-cysteamine or *N*-acetylcysteamine, was used as acyl donor (Table 4), suggesting that the dihydroxydimethylbutyryl part of the phosphopantetheine moiety plays an important role in the proper positioning the substrate in the active site. Replacement of Lys326 with Ala, in the context of the triple mutant, produced an enzyme that exhibited very little activity with any of the model substrates.

Decarboxylation of Malonyl Moieties. The wild-type animal FAS has very limited ability to catalyze the decarboxylation of malonyl moieties, unless a saturated acyl moiety is positioned on the active-site cysteine of the ketoacyl synthase domain in readiness for the ensuing condensation reaction. Replacement of the active-site cysteine with glutamine (Cys161Gln), however, increases the rate of decarboxylation by more than 2 orders of magnitude while completely blocking the condensation reaction (*30*). We have suggested that the side-chain carbonyl group of Gln161 mimics the carbonyl of a saturated acyl moiety in the active site so that the mutant adopts a conformation analogous to that of the normal acyl-enzyme intermediate. The Cys161Gln mutant FAS, therefore, likely provides a useful model that allows identification of residues required for catalysis of the uncoupled decarboxylation reaction at rates compatible with the overall capacity of the wild-type protein for fatty acid synthesis.

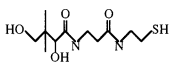
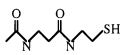
Thus, we compared the effect on decarboxylase activity of replacing the conserved basic residues in the context of the Cys161Gln mutant, as well as the wild-type FAS (Table 5). Replacement of either histidine in the context of Cys161Gln FAS reduced decarboxylase catalytic efficiency by more than 2 orders of magnitude. The decreased efficiency is attributable mainly to a large reduction in the values for k_{cat}, indicating that both histidines likely participate in the catalytic process. Replacement of Lys326 in the context of the Cys161Gln FAS reduced decarboxylase activity by more than 3 orders of magnitude, suggesting that this residue too, plays an essential role in facilitating the decarboxylation reaction. Replacement of His293, His331, or Lys326 with Ala in the context of the wild-type FAS had little effect on the low level of decarboxylase activity that is characteristic of this enzyme in the "resting" state.

Identification of Bicarbonate as a Product of the Malonyl Decarboxylation Reaction. The Cys161Gln FAS was utilized to determine whether carbon dioxide or bicarbonate is released from the enzyme as the final product of decarboxylation. Bicarbonate formation was monitored continuously by coupling with phospho(enol)pyruvate carboxylase, which converts bicarbonate (but not carbon dioxide) and phospho-(enol)pyruvate into oxaloacetate, and malate dehydrogenase, which reduces oxaloacetate to malate, with accompanying oxidation of NADH (*31*). Comparison of initial rates of NADH oxidation in the absence and presence of carbonic anhydrase, which rapidly equilibrates carbon dioxide and bicarbonate, allows identification of the form of carbon dioxide, hydrated or unhydrated, that is released from the FAS. This determination is facilitated by the relatively low rate constant for the noncatalyzed hydration of carbon dioxide, approximately 0.04 s^{-1} at room temperature (*31*). Thus, an enzyme that generates carbon dioxide will reach steady state conditions only after an initial lag, during which the carbon dioxide is converted to bicarbonate. On the other hand, an enzyme that generates bicarbonate will reach steady state without any lag phase.

To avoid interference of the low NADH reduction activity exhibited by wild-type FAS, two FAS mutants that were unable to provide a substrate for the FAS reductases were employed in this experiments. One, C161Q, as mentioned before, lacks condensing activity but can catalyze the uncoupled decarboxylation reaction extremely efficiently.

The second, G1888A FAS, can perform the coupled decarboxylation-condensation reaction as efficiently as the wild-type enzyme; however, due to the mutation in the nucleotide binding site, it cannot contribute to NADH oxidation by reducing the condensation product (data not shown). On addition of either the C161Q or G1888A mutants

Table 4: β-Ketoacyl Synthase-Mediated Acyltransferase Activity of FAS Mutants; Influence of Thiol Donor Structure[a]


FAS	pantetheine			N-acetyl-N-β-alanylcysteamine			N-acetylcysteamine		
	K_m (μM)	K_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
triple mutant[b]	0.06	1.2	20	0.025	0.028	1.12	0.59	0.023	0.39
quadruple mutant (Lys326Ala)	0.046	0.019	0.41	0.023	0.029	1.25	0.043	0.019	0.44

[a] The bidirectional assay system was used with the decanoyl thioester donor substrates indicated and CoASH as the acceptor. [b] Triple mutant: Ser581Ala, Ser2151Ala, Ser2302Ala. Quadruple mutant contains three mutations of the triple mutant and an additional mutation indicated in the parenthesis.

Table 5: Malonyl Decarboxylase Activity of FAS Mutants[a]

FAS background		additional mutations: none	His293Ala	His331Ala	Lys326Ala
		Specific Activity, nmol mg^{-1} min^{-1} (% control)			
wild-type		1.38 (100)	3.23 (233)	1.56 (113)	0.59 (43)
Cys161Gln		520 (100)	6.6 (1.3)	2.6 (0.5)	0.11 (0.02)
		Kinetic Parameters			
Cys161Gln	k_{cat}, s^{-1}	2.82	0.052	0.009	n.d.
	K_m, μM	9.6	33.3	10.8	n.d.
	k_{cat}/K_m, $\mu M^{-1}\ s^{-1}$	0.294	0.002	0.001	

[a] Assays were performed with malonyl-CoA at 110 μM CoASH at 50 μM. n.d., not determined.

to the reaction mixture, the initial rate of bicarbonate production was identical in the absence and presence of carbonic anhydrase, indicating that bicarbonate, not carbon dioxide, was the end product of the malonyl decarboxylation reaction (Figure 3A and 3B). In contrast, pyruvate decarboxylase, an enzyme that releases carbon dioxide as the product (*31*), when added to the reaction mixture, produced a distinct lag of about 1.5 min before reaching steady-state reaction conditions (Figure 3C), as has been reported previously.

The calculated rate of formation of bicarbonate dependent on the presence of malonyl-CoA (68 ± 3 nmole min^{-1} mg^{-1}) in the steady-state phase was identical to the rate of acetyl-CoA formation from malonyl-CoA, 71 ± 4 nmole min^{-1} mg^{-1}, as measured independently by hplc for the C161Q FAS, indicating that all decarboxylated malonyl could be accounted for as acetyl-CoA and bicarbonate.

In an attempt to determine whether one of the two histidines required for catalysis of the decarboxylation reaction might interact with the leaving carboxylate of the malonyl moiety, we hypothesized that bicarbonate, the reaction product, might be an effective inhibitor of such an interaction, and therefore a potentially useful probe. Indeed, sodium bicarbonate proved to be a competitive inhibitor of malonyl in the decarboxylation reaction catalyzed by the Cys161Gln FAS mutant at pH 6.6 with an IC_{50} of 11.5 mM (Figure 4). The reaction was only weakly inhibited by sodium acetate (IC_{50}:61 mM), suggesting that a protonated carboxyl is required for effective competition with malonyl moieties. Replacement of His293 or His331 with alanine, in the context of the Cys161Gln FAS, produced different effects. Whereas introduction of the His293Ala mutation reduced sensitivity to inhibition by bicarbonate to a level (IC_{50}:63 mM) comparable with that observed for acetate, introduction of the His331Ala mutation had less effect (IC_{50}: 33 mM).

This result indicated that it is His293 that is most likely to interact with the 3-carboxylate of the malonyl substrate. Wild-type FAS, which exhibits extremely low decarboxylase activity, was inhibited very weakly by bicarbonate (IC_{50}:77 mM), consistent with our earlier suggestion that, in the absence of an acyl moiety covalently bound to Cys161, the wild-type enzyme adopts a conformation that does not permit proper binding of the malonyl moiety.

Fatty Acid and β-Ketobutyryl-CoA Synthesis. Amino acid replacements were introduced at each of the highly conserved positions, His293, His331, and Lys326, and the effect on the ability of the FAS to catalyze the condensation reaction was assessed. Two different assay systems were employed, using acetyl- and malonyl-CoA as substrates: [i] the overall FAS reaction, in the presence of NADPH, that includes substrate loading, iterative cycles of condensation and intermediate β-carbon processing, and the release of the fatty acid product and [ii] a simplified β-ketobutyryl synthesis/condensation assay, in the absence of NADPH, that measures only substrate loading and a single condensation reaction, without any involvement of the normal β-carbon processing and chain terminating reactions. In the latter assay, the β-ketobutyryl moiety formed in the condensation reaction is released by direct transfer to a CoASH acceptor (*25*). Replacement of either of the conserved histidine residues, or the conserved lysine residue, with alanine generated

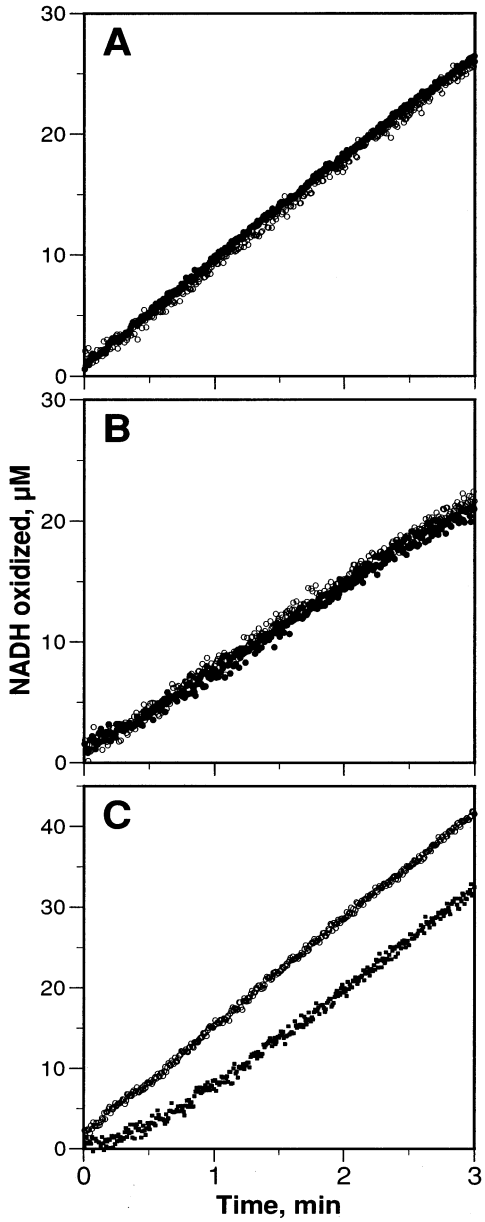


FIGURE 3: Progress curves of bicarbonate production by (A) C161Q FAS mutant from malonyl-CoA, (B) G1888A FAS mutant from acetyl- and malonyl-CoA, and (C) pyruvate decarboxylase from sodium pyruvate in the presence (open circles) and absence (closed circles) of carbonic anhydrase. The first point was recorded 7−8 s after the start of the reaction.

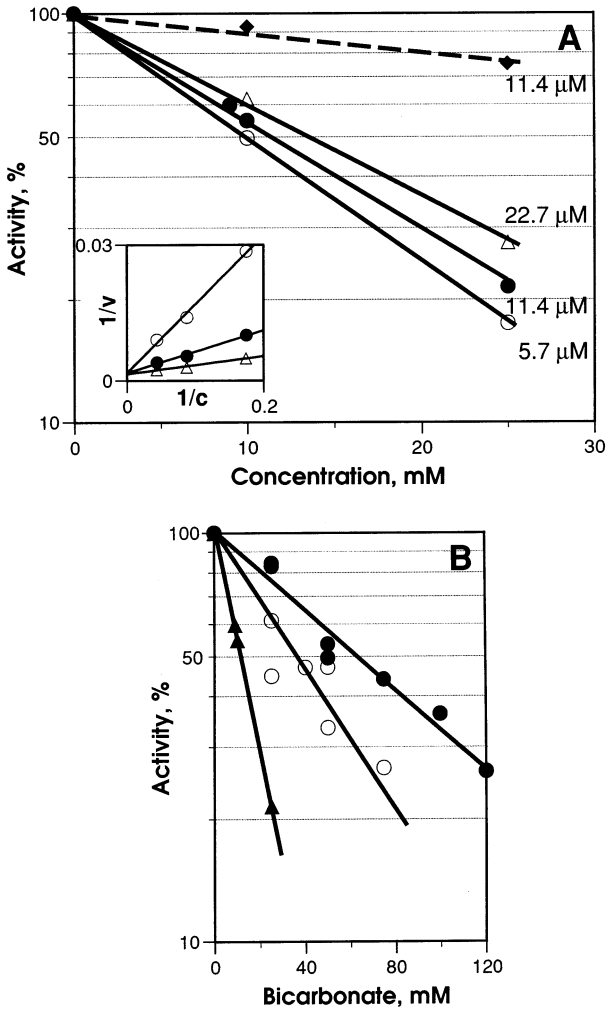


FIGURE 4: Inhibition of decarboxylation of [2-^{14}C]malonyl-CoA by sodium bicarbonate. (A) Cys161Gln FAS single mutant (0.17 μg) was incubated with [2-^{14}C]malonyl-CoA at indicated concentrations in the presence of 50 μM CoASH and either bicarbonate (solid lines) or acetate (dashed line) at 37 °C for 1 min. (B) Cys161Gln,His293Ala or Cys161Gln,His331Ala FAS double mutants (21.5 μg) were incubated with 11.4 μM [2-^{14}C]malonyl-CoA in the presence of 50 μM CoASH and bicarbonate at 37 °C for 1 min. Bicarbonate IC_{50} values were 11.5 mM for Cys161Gln (triangles), 63 mM for Cys161Gln,His293Ala (closed circles), and 33 mM for Cys161Gln,His331Ala (open circles). The acetate IC_{50} value for Cys161Gln was 61 mM.

mutants with significantly reduced activity in both assays (Table 6). Replacement of both histidine residues, in the double mutant His293,His331Ala, reduced activity in the condensation assay by more than 3 orders of magnitude. Mutation of either, or both, of the two histidines shifted the pH optimum from 6.6 to 6.0. The pH dependence of β-ketobutyryl synthesis catalyzed by the wild-type enzyme (Figure 5) closely resembles that for fatty acid synthesis by native rat FAS (*40*). In quantifying, by hplc, the amount of [2-^{14}C]β-ketobutyryl-CoA formed in the single cycle condensation assay, we noticed that several mutants also generated radiolabeled acetyl-CoA.

Radiolabeled acetyl-CoA can be generated only by uncoupled decarboxylation of [2-^{14}C]malonyl moieties by the β-ketoacyl synthase domain and release of the [2-^{14}C]acetyl moiety by transfer to CoA. Thus, wild-type FAS released only β-ketobutyryl-CoA as product; no [2-^{14}C]acetyl-CoA was produced, indicating that decarboxylation and condensation were tightly coupled. In contrast, all of the mutant FASs released significant amounts of [2-^{14}C]acetyl-CoA, indicating that utilization of carbanion in the condensation step was rate limiting.

DISCUSSION

The first step in the reaction sequence catalyzed by the β-ketoacyl synthase family of enzymes involves a nucleophilic attack by the active-site cysteine residue on the substrate carbonyl. The −SH group is a poor nucleophile, and formation of the nucleophilic deprotonated thiol form in the pH range of maximal activity would normally be limited by the high pK_a for the thiol group, ~9. For a number of years, it has been generally assumed that nucleophilicity of the cysteine thiol likely is promoted by an adjacent

Table 6: Ability of FAS Mutants to Catalyze Single and Multiple Rounds of Condensation

	FAS				
assay	wild type	His293Ala	His331Ala	His293Ala and His331Ala	Lys326Ala
	Specific Activity, nmol mg^{-1} min^{-1} (% wild-type activity)				
single[a]	83.1 (100)	11.0 (13.2)	2.8 (3.4)	0.04 (0.05)	0.10 (0.12)[c]
multiple[b]	1970 (100)	88 (4.5)	16 (0.8)	0.04 (0.002)	≤10 (0.5)

[a] The assay system for β-ketobutyryl-CoA synthesis was used. [b] The assay system for overall fatty acid synthesis was used. [c] Data taken from (*25*).

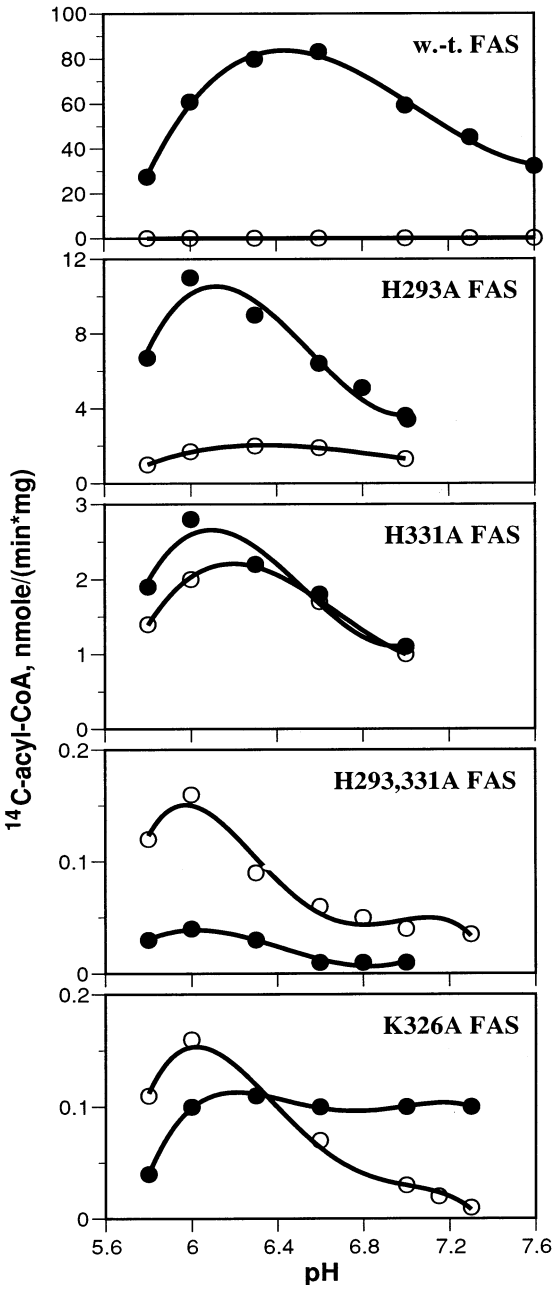


FIGURE 5: pH dependency of the single condensation reaction; release of β-ketobutyryl-CoA and acetyl-CoA. Reaction mixtures, containing 50 μM acetyl-CoA, 95 μM [2-^{14}C]malonyl-CoA, 100 μM CoASH, enzyme, and 0.2 M phosphate buffer, at indicated pH, were incubated at 37 °C for 2 min. No triacetic acid lactone is formed under these conditions. (Filled circles, β-ketobutyryl-CoA; open circles, acetyl-CoA.)

histidine residue that acts as a general base, extracting the proton from the active cysteine thiol and lowering its pK_a (*17*, *41*−*43*). Examination of the crystal structures of β-ketoacyl synthase I (*18*), II (*19*), and III (*20*−*22*) from *E. coli* and β-ketoacyl synthase II from *Synechocystis sp.* (*23*) appeared to support this view; in all four crystal structures, the conserved histidine (His333, His340, His244, and His344, respectively) is positioned within hydrogen-bonding distance from the cysteine nucleophile. Nevertheless, when the proposed role of the conserved histidine was tested by mutagenesis in the *E. coli* enzymes, the results were contradictory, since the His333Ala mutant in β-ketoacyl synthase I (*44*) and the His244Ala mutant in β-ketoacyl synthase III (*21*) exhibited enhanced, rather than depressed, acyltransferase activity. Davies et al. (*21*) suggested an alternative mechanism for activation of the cysteine nucleophile, namely, that the active-site cysteine residue, located at the N-terminus of an α-helix, benefits from the positive charge generated by the helix-dipole effect, significantly lowering its thiol pK_a. Our mutagenesis experiments with the β-ketoacyl synthase domain of the animal FAS indicate clearly that reactivity of the active-site residue Cys161 toward iodoacetamide is unaffected by replacement of the His331 residue positionally equivalent to His333, His340, and His244 in the *E. coli* β-ketoacyl synthases I, II, and III, respectively. Nor is the reactivity of Cys161 dependent on the presence of His293, which is positionally equivalent to residues His298 and His303 in the *E. coli* β-ketoacyl synthases I and II, respectively, which are also located close to the cysteine nucleophile. On the contrary, the reactivity of the active-site cysteine residue toward iodoacetamide is actually increased approximately 20-fold upon replacement of either of the histidine residues with alanine. Possibly the increased rate of alkylation results from easier access of the reagent to the nucleophile as a result of replacement of the histidyl side chains with the less bulky alanyl side chain. Although the possibility that the somewhat unexpected results of histidine replacement experiments in both the type I and type II β-ketoacyl synthases may have resulted from subtle, undetected changes in enzyme conformation brought about by the introduction of the mutations cannot be formally excluded, it seems unlikely, since the replacements uniformly result in increased reactivity of the active-site cysteine residue. Furthermore, when either of the two conserved histidine residues was replaced by alanine, the pH optimum for the overall condensation reaction actually decreased. If either of the histidine residues was responsible for lowering the pK_a of the cysteine nucleophile, one would have anticipated the mutants would have exhibited an increase in the pH optimum. Sequence alignments and secondary structure predictions (not shown) indicate that, in common with type II β-ketoacyl synthases, the active-site cysteine residue of the animal counterpart is located in at the aminoterminus of an α-helix that immediately follows a

β-sheet. This motif has been termed a "nucleophilic elbow" (*45*, *46*). Thus, our findings are consistent with the concept that activation of the cysteine nucleophile results largely from exploitation of a helix dipole moment, as first proposed for the *E. coli* β-ketoacyl synthase III. A similar conclusion has been reached recently based on structural studies on the *E. coli* β-ketoacyl synthase I (*46*). Catalysis of the acyltransfer reaction and formation of the covalent acyl-enzyme intermediate, then, is promoted primarily as a result of the lowered pK_a of the Cys161 nucleophile and activation of the substrate thioester carbon atom by binding of the keto group in the oxyanion hole.

Mutation of the conserved histidine residues in *E. coli* β-ketoacyl synthase I and the animal FAS, or the positionally equivalent Asn274 in *E. coli* β-ketoacyl synthase III, does not significantly compromise acyltransferase activity, implying that none of these residues participates in formation of the oxyanion hole. The three-dimensional structures of the fatty acid complex with *E. coli* β-ketoacyl synthases I (*47*), the cerulenin complex with *E. coli* β-ketoacyl synthase II (*48*), and the structures of *Synechocystis* β-ketoacyl synthase II (*23*) and *E. coli* β-ketoacyl synthase III (*20*−*22*) all strongly suggest that the negatively charged transition state is likely stabilized by interactions between the substrate carbonyl and the backbone amides of the active-site cysteine and either a conserved phenylalanine, in β-ketoacyl synthases I and II, or glycine in β-ketoacyl synthases III, near the C-terminus (Figure 1). The region immediately adjacent to the conserved phenylalanine/glycine is characteristically glycine-rich, a feature thought to be essential to allow access of substrate into the oxyanion hole. Although, in the absence of a crystal structure for the β-ketoacyl synthase domain of the animal FAS, it is not possible to identify with certainty the location of the oxyanion hole in the animal enzyme, the highly conserved nature of the glycine-rich region provides a strong indication that it is the backbone amide of Phe395, together with that of the active-site cysteine residue, that stabilizes the substrate carbonyl in the transition state. This hypothesis is further supported by characterization of the animal FAS Cys161Gln mutant, together with modeling studies performed on *E. coli* β-ketoacyl synthase II, which predicted that, in the animal FAS, the backbone amide of Phe395 contributes to the binding pocket normally reserved for the substrate carbonyl, but that in the Cys161Gln mutant this oxyanion hole is occupied by the side-chain carbonyl of the glutamine residue (*30*).

Mutagenesis of *E. coli* β-ketoacyl synthase I (*44*) indicates that both His298 and His333 are required for catalysis of the malonyl decarboxylation reaction. Similarly, replacement of the equivalent residues in *E. coli* β-ketoacyl synthase III indicates that both His244 and Asn274 play essential roles in the decarboxylation reaction (*21*). Nevertheless, despite the availability of three-dimensional structures for these enzymes and the β-ketoacyl synthase II enzymes from *E. coli* and *Synechocystis*, there is no clear consensus as to the precise roles played by these residues. On the basis of analysis of the crystal structure of the *E. coli* β-ketoacyl synthase I-thiolactomycin[3] complex, the Memphis group (*49*) proposed that the thioester carbonyl oxygen is hydrogen bonded to both His298 and His333, promoting formation of the carbanion at C-2 of malonyl. The same team earlier proposed a similar mechanism for decarboxylation of malonyl moieties by the *E. coli* β-ketoacyl synthase III; in this case the two residues involved were His244 and Asn274. On the other hand, by modeling a malonyl thioester into their crystal structure of *E. coli* β-ketoacyl synthase I, the Copenhagen group deduced that whereas His333 does indeed contact the thioester carbonyl oxygen, His298 likely acts as a general base by extracting a proton from the leaving carboxylate at C-3 (*47*). Both of these proposed mechanisms are based on the assumption that carbon dioxide is released as the product. Our direct, quantitative measurement of the reaction products indicates that, at least for the β-ketoacyl synthase associated with the animal type I FAS, the malonyl C-3 is actually released as bicarbonate. This inference cannot result from perturbation of the active center by the C161Q mutation, used to study the uncoupled rate of malonyl decarboxylation, since identical results were obtained using the G1888A FAS, which contains a "wild-type" ketoacyl synthase domain and catalyzes the overall condensation reaction at the same rate as that of wild-type FAS. Furthermore, bicarbonate is a competitive inhibitor, the effectiveness of which is eliminated in the His293Ala mutant but not in the His331Ala mutant. Although this differential effect of the two histidine mutations on sensitivity to bicarbonate appears compatible with the Copenhagen model, as illustrated in Figure 6A, this mechanism would require inclusion of a post decarboxylation step for hydration of the carbon dioxide after decarboxylation. We suggest an alternative mechanism, compatible with our experimental data, can be contemplated that would result in the direct release of bicarbonate as product (Figure 6B). In this mechanism, the carboxylate at C-3 is not protonated, as assumed in the mechanism depicted in Figure 6A, but the negative charge is partially neutralized by interaction with His293. The reaction is initiated by attack of an activated water molecule, or hydroxide, on the malonyl C-3.

The resulting negatively charged tetrahedral transition state, stabilized by interaction of His293 with a negatively charged oxygen at C-3 and interaction of His331 with the negatively charged thioester carbonyl oxygen, would break down to generate the acetyl carbanion and bicarbonate directly.

In the final step of the reaction sequence, the carbanion, formed by decarboxylation of the malonyl moiety, attacks the thioester carbon of the fatty acid bound to Cys161, and a new C−C bond is formed. It is difficult to evaluate independently the role of specific residues in this process, since any assay system inevitably requires that the acyltransfer and decarboxylation steps precede the condensation event. Nevertheless, by examining the products formed when the enzyme is presented with both acetyl and malonyl substrates, it is possible to identify indirectly those residues that are essential for catalysis of the condensation reaction. In the absence of NADPH, the first condensation product, a β-ketobutyryl-S-phosphopantetheine thioester, is translocated to free CoASH in a reaction catalyzed by the malonyl/acetyltransferase domain of the FAS. Indeed, β-ketobutyryl-CoA is the sole product formed by wild-type FAS in the "single cycle" assay system (Figure 5). However, all of the mutants studied released radiolabeled acetyl-CoA together with β-ketobutyryl-CoA. The release of radiolabeled acetyl

[3] Thiolactomycin mimics the noncovalently bound thiomalonate.

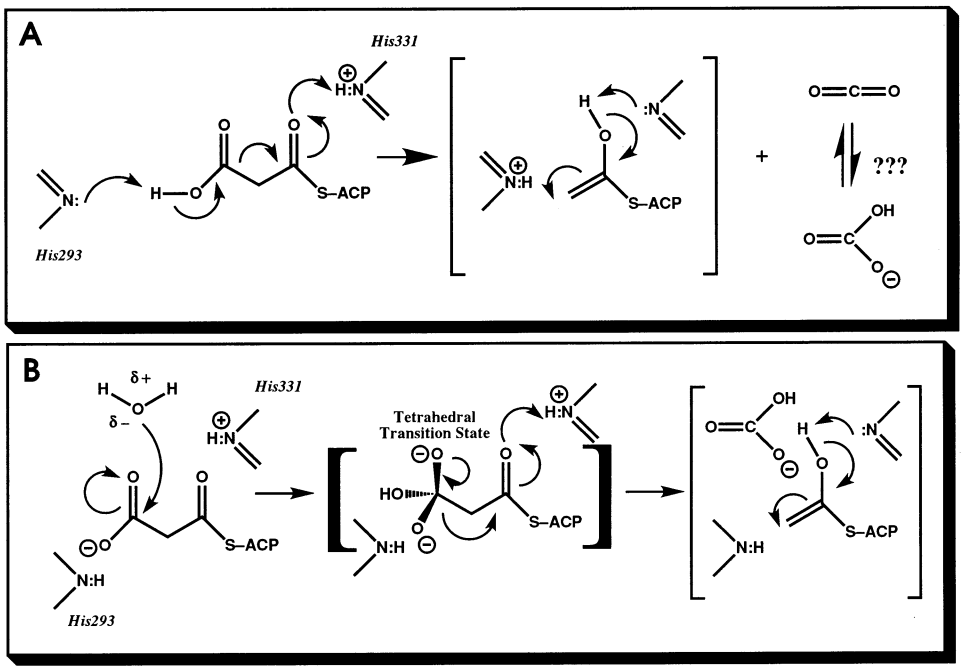


FIGURE 6: Alternative mechanisms for the malonyl decarboxylation reaction.

moieties, produced by decarboxylation of radiolabeled malonyl moieties, indicates that the decarboxylation and condensation reactions are partially uncoupled in these mutants. Thus, a significant proportion of the radiolabeled acetyl carbanions produced react with a proton, rather than with the thioester carbon of the unlabeled acetyl moiety bound to Cys161, and the product is released by translocation to CoASH. The extent of this "leakage" of the decarboxylation product is relatively small in the case of the His293Ala mutant but particularly noticeable in the case of the His331Ala mutant. The implication of these findings is that His331 appears to play a role in condensation reaction. Most likely then, both the malonyl moiety and the carbanion decarboxylation product are stabilized by interaction of the thioester carbonyl with His331. Similarly, the oxyanion hole continues to play a role in the condensation reaction by stabilizing the transition state tetrahedral intermediate. Finally, the transition state intermediate breaks down, and the β-ketobutyryl-S-phosphopantetheine thioester is formed. In the absence of NADPH, this intermediate is translocated to CoASH (single cycle assay), but in the presence of NADPH, the β-carbon processing reactions follow and additional rounds of chain elongation can occur.

Assigning a functional role for the conserved lysine residue present in the animal FAS and the β-ketoacyl synthases I and II (Figure 1) has proven difficult. In the crystal structure of the *E. coli* β-ketoacyl synthase II, the side chain of Lys335 points away from the Cys163 thiol, some 7.8 Å away, prompting Huang et al. (*19*) to suggest that the role of this lysine might be structural, rather than catalytic. However, crystallographic analysis of *E. coli* β-ketoacyl synthase I indicates that in this enzyme, Lys328 is part of a hydrogen bonding network at the active center (*47*). In this model, the imidazole of His298 is linked with the amino group of Lys328 by a hydroxide, which, in turn, is hydrogen bonded to a water molecule. This interaction may enable His298 to act as a general base in promoting the decarboxylation reaction.

While the results of our own studies certainly do not rule out the possibility that in the animal FAS the role of Lys326 in initiating the decarboxylation reaction by activation of His298, as suggested for *E. coli* β-ketoacyl synthase I, neither are they incompatible with a possible structural role for this residue. The observation that the Lys326Ala mutant retains significant acyltransferase activity in the unidirectional assay, but not in the bidirectional assay is intriguing. We earlier proposed that binding of the first substrate to the Cys161 nucleophile induces a conformational change that facilitates access by the second substrate, the malonyl-S-phosphopantetheine thioester (*30*). It is possible that, in the bidirectional acyltransferase assay, once the covalent acyl-S−Cys161 intermediate has formed, the same conformational change is required to permit access of the pantetheine thiol acceptor. Thus, one might speculate that in the case of the Lys326Ala mutant, this conformational change cannot readily take place, so the bidirectional transfer of acyl moieties, but not the unidirectional transfer, is compromised.

The inhibition of β-ketoacyl synthase-mediated acyltransferase activity achieved with apparent substoichiometric *S*-carboxamidomethylation of the Cys161 nucleophile and the substoichiometric saturation achieved in the acyl transfer reaction with a variety of substrates are puzzling observations. The possibility that removal of dithiothreitol, prior to treatment with iodoacetamide, could cause partial oxididation of the C161 thiol can discounted for three reasons. First, we have used the same procedure for removal of dithiothreitol previously and found that more than 95% of the Cys161 thiol could be reacted with dibromopropanone (*50*). Second, we included tris(2-carboxyethyl)phosphine as a reducing agent in the *S*-carboxamidomethylation experiments. Third, we found no loss in acyltransferase activity after removal of dithiothreitol. Substoichiometric loading of single substrates by type I FASs is commonly observed but poorly understood. Thus, the available substrate binding sites of neither the animal nor the yeast FASs can be fully loaded, even in the presence of saturating substrate concentrations; this is despite

the fact that, under steady-state conditions, both types of FAS exhibit full-sites active characteristics (*51*, *52*). Clearly, further experimentation is required to examine the possibility that the two β-ketoacyl synthase active sites present in the FAS dimer may be interactive.

A common feature of β-ketoacyl synthase isolated from plants and microorganisms is that they are all active as homodimers. Crystallization of β-ketoacyl synthases I, II, and III as dimers has revealed that the substrate binding sites are comprised of residues from both subunits. Although the animal FAS too is a homodimer, there is no information available as to whether the β-ketoacyl synthase domains themselves participate in homodimeric interactions. Indeed, earlier models visualized the two subunits oriented in a fully extended antiparallel orientation, precluding this possibility. More recently however, mutations at the active center of the β-ketoacyl synthase domain have been identified that markedly affect stability of the FAS dimers, and a considerable body of evidence has accumulated that questions the antiparallel subunit model. Resolution of the question as to whether the remarkable similarity between the type I and II β-ketoacyl synthases extends to the level of quaternary structure most likely will require characterization of an animal β-ketoacyl synthase domain expressed as an independent protein.

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