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The Conserved Serine–Threonine–Serine Motif of the Carnitine Acyltransferases Is Involved in Carnitine Binding and Transition-State Stabilization: A Site-Directed Mutagenesis Study[1]

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There has been speculation that the carnitine acyltransferase reaction mechanism may involve the formation of an acyl-serine intermediate. A serine–threonine–serine (STS) motif that is conserved throughout the carnitine acyltransferase family, and is present also in the choline acetyltransferases, contains the only two conserved serines. The functional role of this motif in carnitine octanoyltransferase was probed by using a site-directed mutagenesis strategy to generate all seven possible alanine substitutions: single, double and triple mutants. Kinetic analyses of these mutant enzymes demonstrated that the STS motif is not essential for catalysis, thereby excluding an acyl-serine intermediate from the reaction mechanism. The kinetic analyses support, however, substantial roles for the STS motif in carnitine binding and transition-state stabilization.

The carnitine acyltransferases catalyze the reversible transfer of variable length fatty acyl groups between CoA and carnitine, and play a central role in fatty acid metabolism (for a review of these enzymes see (1)). Although the cellular roles of the various carnitine acyltransferases are generally well established, little is known regarding the mechanism of the acyltransferase reaction. In addition, although the cDNAs for about ten carnitine and five choline acyltransferases have been cloned and the deduced amino acid sequences reported (see references within), a three-dimensional structure has not yet been described for any of these enzymes.

Early experiments (2) involving the use of the active site-directed reagent bromoacetyl-L-carnitine suggested that a histidine residue is involved in the acyltransferase reaction mechanism, and led to speculation that the catalytic process might involve an Asp-His-Ser triad, analogous to the serine protease group of enzymes, with which to form an acylated enzyme intermediate. However, kinetic studies with pigeon breast carnitine acetyltransferase (CAT) (3) and, more recently, with bovine liver carnitine palmitoyltransferase-II (CPT-II) and carnitine octanoyltransferase (COT) (4) suggest that these enzymes obey reaction mechanisms that involve ternary complex formation, compulsory-ordered for CPT-II and rapid-equilibrium random-ordered for CAT and COT. Such findings are inconsistent with a process involving a free modified enzyme intermediate. Recently, Brown *et al.* (5) suggested that the carnitine acyltransferase reaction mechanism may indeed involve a catalytic triad but one in which the hydroxyl group at C3 of carnitine replaces the archetypal hydroxyl of serine to act as the nucleophile toward the acyl-CoA substrate.

All carnitine acyltransferases contain a conserved Ser-Thr-Ser (STS) motif near their carboxy termini that appears to contain the only two conserved serine residues. This motif is present also in the choline acetyltransferases, although these enzymes contain additional conserved serine residues. The present report describes the results of a site-directed mutagenesis study that targets this motif in carnitine octanoyltransferase and demonstrates that neither of these two con-

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Abbreviations: AAA-, AAS-, ATA-, ATS-, SAA-, SAS- and STA-COT, mutant forms of bovine carnitine octanoyltransferase in which the STS motif at residues 473–475 has been substituted with alanine as indicated; CAT, carnitine acetyltransferase; ChAT, choline acetyltransferase; COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; STS, conserved serine–threonine–serine motif present in the carnitine and choline acyltransferases.

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served serines are essential for catalytic activity, thereby excluding an acyl-serine intermediate from the carnitine acyltransferase reaction mechanism. The data support, however, significant roles for each of the residues within the STS motif in carnitine acyltransferase catalysis.

MATERIALS AND METHODS

Materials. All biochemicals and protein purification materials used in this study were obtained from the sources described previously (6). 5-bromo-4-chloroindolyl phosphate and nitroblue tetrazolium were obtained from Sigma. The construction of pTHbCOT, an *E. coli* expression vector for the production of recombinant bovine COT, and the procedures used to purify the recombinant enzyme were described previously (6). Anti-bovine COT polyclonal antibody #501 was kindly provided by Dr N. Nic a'Bháird and Prof. T. P. Singer.

Site-directed mutagenesis of the STS motif in bovine COT. Substitutions with alanine at the STS motif (residues 473-475) in bovine COT were carried out by using the same target template, selection primer and general procedures described previously (6). In this case, the mutagenic oligonucleotide was oCOT-STS [5′-GGA.GGA.GGT.GGA. AAC. TTT. GTG. CTA. KCA. RCA. KCG. CTG. GTT. GGT. TAT. TTA. AGA.GTC.CAG.GGC-3′; where K represents G or T, R represents A or G and the underlined bases are mutagenic], and was designed to generate all seven single, double and triple mutants, in addition to the wild type sequence (see Fig. 2 for strategy). The AAA-COT triple mutant (see Results section for nomenclature) was the only mutant not obtained after analysis of 81 progeny from the mutagenesis procedure described above and this mutant was generated in a separate site-directed mutagenesis reaction by using the specific oligonucleotide oCOT-AAA [5′-GGA.GGA.GGT.GGA.AAC.TTT.GTG.CTA.GCA. GCA.GCG.CTG.GTT.GGT.TAT.TTA.AGA.GTC.CAG.GGC-3′].

The cloned inserts of selected mutants were sequenced on both DNA strands in their entirety and the *Hin*d III/*Eco*R I mutant inserts used to replace the wild type fragment in the pTHbCOT expression vector. The expression construct of each mutant was verified by restriction mapping and, in the case of those mutants that did not have novel restriction sites introduced, by sequencing across the mutated site using oligonucleotide oCOT3 (5′-CAA.TCC.ATG.CAG.AAT. CCT.TCT.ACC-3′; sequence encodes residues 467-474 of the bovine COT structural gene) as primer. DNA sequence analysis was carried out as described previously (6). The wild type and mutant COT enzymes were expressed in *E. coli* DH5αF′IQ (Gibco-BRL).

Preparation of E. coli extracts containing COT mutants. Crude extracts of *E. coli* containing COT enzymes mutated at the STS motif were prepared as follows: a 1 ml inoculum of *E. coli* DH5αF′IQ harboring the mutant expression vector that had been grown overnight at 37°C and 300 rpm in 3 ml of Luria-Bertani medium containing 100 μg/ml ampicillin was added to 100 ml of Luria-Bertani medium without ampicillin and growth was continued at 30°C and 300 rpm. When the A_{550} of the culture reached 0.5-0.7, isopropyl thio-β-D-galactoside was added to a final concentration of 0.1 mM and growth continued overnight (15-18 h). The culture was centrifuged at 4,000*g* for 10 min at 4°C, the supernatant discarded and the whole cell pellet resuspended in 2 ml of 5×TED buffer (1×TED buffer is 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (free acid) and 0.2 mM dithiothreitol). The cell suspension was sonicated on ice by using a microtip connected to a SONIFER Cell Disruptor W185 (Heat Systems-Ultrasonics) at setting 6 for 10 s on/2 min off and for a total of six times. The suspension was centrifuged at 10,000*g* for 10 min at 4°C and the supernatant dialyzed against 2 L of TED buffer for 4 h at 4°C. Measurements of COT activity in the dialyzed extracts were carried out as described below and were conducted on the same day as the extracts were prepared.

The AAA-COT triple mutant was purified to near-homogeneity in a manner similar to that described previously for the recombinant wild type enzyme (6). The concentrations of purified COT enzymes were determined by using an extinction coefficient at 280 nm of 1.00 $\times$ 10^5 $M^{-1}cm^{-1}$(6).

Western blotting. Protein samples that had been fractionated by electrophoresis through 10% SDS-PAGE slab gels as described previously (6) were transferred to Hybond-C nitrocellulose membranes (Amersham) by using a Mini-Protean Transblotter (Bio-Rad) according to the manufacturers instructions. Following transfer, the lane containing the standard molecular weight marker proteins was dissected away and stained for protein by immersion for 2 min in a 0.1% (w/v) amido black solution prepared in 45% (v/v) methanol/10% (v/v) acetic acid, followed by storage in a small volume of water. Western blot analysis was carried out on the remainder of the filter as described in (7) and by using 1% (w/v) bovine serum albumin (Sigma A7906) in phosphate buffered saline as blocking reagent. Filters were probed with polyclonal antibody #501 (1:500 dilution) that had been raised against a sample of COT purified from bovine liver. The binding locations of the primary antibody were determined subsequently by probing with a goat anti-rabbit IgG (H+L) secondary antibody conjugated to alkaline phosphatase (Bio-Rad) at 1:2,000 dilution and developed with 5-bromo-4-chloroindolyl phosphate and nitroblue tetrazolium.

Measurement of COT activity. COT activity was determined as described previously (6). The activity of COT in crude extracts of *E. coli* was corrected for the presence of an endogenous acyl-CoA hydrolase activity by measuring the rate of decanoyl-CoA hydrolysis in the absence of carnitine. During the purification of the AAA-COT triple mutant, the assay concentration of carnitine was 10 mM. One unit (u) of enzyme activity is defined as the amount of enzyme required to catalyze the conversion of 1 μmole of substrate to product in 1 min. Substrate saturation data were fitted to rectangular hyperbolae by non-linear regression by using the computer program Enzfitter (Biosoft, UK).

RESULTS

All carnitine acyltransferase sequences contain a conserved STS motif, shown in Fig 1, near their carboxy termini that appears to contain the only two conserved serine residues. This motif is present also in the choline acetyltransferases (see Fig. 1) although, during routine protein alignment with CLUSTAL V (17), this motif does not align among all sequences because the CPT-I sequences are pushed slightly out-of-phase. It was considered that the replacement of these residues in COT with alanine might help to resolve the issue of whether or not the carnitine acyltransferase reaction mechanism makes use of a catalytic triad that involves a participating serine residue.

The conservation of the STS motif suggested, however, that even if the residues within this sequence were not involved in the formation of a catalytic triad, they might serve some alternative critical function and, thus, that any mutagenesis protocol adopted to investigate this motif should be subtle in its approach. Therefore, a site-directed mutagenesis strategy was developed, as shown in Fig. 2, that would allow for the generation of all possible combinations of alanine substitutions within the STS motif i.e. single, double and triple mutants, by using a single mutagenic oligonucle-

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                                                                  •••
Human CPT I      654 DRHLFCLYVVSK--YLAVESPFLKEVLSEP-----WRLSTSQTPQQQVELFDLENNPEYVSSGGGFGPVADDGYGVSY 724
Rat CPT I        654 DRHLFCLYVVSK--YLAVDSPFLKEVLSEP-----WRLSTSQTPQQQVELFDFEKNPDYVSCGGGFGPVADDGYGVSY 724
Rat CPT I-like   654 DRHLFCLYIVSK--YLGVRSPFLDEVLSEP-----WSLSTSQIPQFQICMFDPKQYPNHLGAGGGFGPVADHGYGVSY 724
Human CPT II     553 DRHLFALRHLAAAKGIILPELYLDPAYGQI---NHNVLSTSTLSSPAVNL-------------GGFAPVVSDGFGVGY 614
Rat CPT II       553 DRHLYALRYLATARGLNLPELYLDPAYQQM---NHNILSTSTLNSPAVSL-------------GGFAPVVPDGFGIAY 614
Mouse CPT II     553 DRHLFALRYLAAARGVTLPELYQDPAYQRI---NHNILSTSTLSSPAVSL-------------GGFAPVVPDGFGIAY 614
Rat COT          504 DRHLLGLLLIAKEEGLPVPELFEDPLFSRSGGGGNFVLSTSLVGYLRIQ------------GVV--VPMVHNGYGFFY 567
Bovine COT       504 DRHLLGLSLIAKEEGLPVPELFTDPLFSRSGGGGNFVLSTSLVGYLRVQ------------GVM--VPMVHNGYGFFY 567
Mouse CAT        518 DRHLLGLKLQAIEDLVSMPDIFMDTSYAIAM---HFNLSTSQVP-AKTD------------CVMFFGPVVPDGYGICY 579
Pigeon CAT       518 DRHLLGLKLQAIEDLVSIPELFMDTAYAVAM---HFNLSTSQVP-AKTD------------CVMCFGPVVPDGYGICY 579
Yeast CAT        559 DRHFFGLKNMLKSNDDQIPPLFKDPLFNYS---STWLISTSQLS---SEYFD----------GYGWSQVNDNGFGLAY 620
                     ***.  *          .  .              .***                          .  .*.*  *

                                                                  •••
Human ChAT       622 NHLLAL----RELARAMCKELPEMFMDETYLMSNRFVLSTSQVPTTTEM---FCCYGPVVPNGYGACYNPQPETILFC 692
Rat ChAT         514 NHLLAL----RELARDLCKEPPEMFMDETYLMSNRFVLSTSQVPTTMEM---FCCYGPVVPNGNGACYNPQPEAITFC 584
Pig ChAT         515 NHLLGL----RELAREVCKELPEMFTDETYLMSNRFVLSTSQVPTTMEM---FCCYGPVVPNGYGACYNPQPESILFC 585
C.eleg ChAT      518 NHLCALFCLAREREETTGEDIPSLFLDPLWSEVMRFPLSTSQVTTSLDIPDCYLTYGAVVRDGYGCPYNIQPDRVIFA 595
D.mel ChAT       618 IPLLGL----REASIEVTGEMHELFKDESYNISQCFLLSTSQVACSTDS---FMGYGPVTPRGYGCSYNPHPEQIVFC 688
                       * .*    **        .   .* *        * ******  . .    .  **.*   * *  ** .*. . *
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FIG. 1. Conservation of the STS motif among the carnitine and choline acyltransferases. The amino acid sequences of human CPT-I (8; L39211), rat CPT-I (9; L07736), rat CPT-I-like (10; D43623), human CPT-II (11; M58581), rat CPT-II (12; J05470), mouse CPT-II (13; U01163), rat COT (14; U26033), bovine COT (6; U65745), mouse CAT (15; X85983), pigeon CAT (16; U08229) and yeast CAT (Kispal, G., Tomcsanyi, T., Sumegi, B., Bock, I., Gajdos, G., Dietmayer, K., and Sandor, A., 1992; Z14021) were aligned using Clustal V (17). The amino acid sequences of human ChAT (18; S56138), rat ChAT (19; P32738), pig ChAT (20; M27736), *C. elegans* ChAT (21; L08969) and *D. melanogaster* ChAT (22; M63724) were aligned separately. The portions of these alignments that contain the conserved STS motif are shown. All accession numbers indicated are GenBank entries with the exception of rat ChAT, which is a Swiss Protein database entry. The residue numbers encompassing each sequence portion are indicated and, within each alignment, an asterisk (*) identifies a residue that is identical in all sequences and a period (.) a residue that is conservatively substituted among all sequences. The conserved STS motif is indicated in both alignments with the bullet points.

otide. The design features included a mechanism for the segregation of the mutant progeny into four pools of two different mutants per pool (including wild type) by performing a simple restriction enzyme analysis and, also, that individual mutants could be "fingerprinted" by performing a single-lane 'G'-track sequencing reaction (see the legend to Fig. 2 and the MATERIALS AND METHODS section for additional details).

A total of 81 mutant progeny were screened and, following *Nhe* I and *Aor*51H I (*Eco*47 III isoschizomer) restriction enzyme analysis, were segregated into four pools of mutant pairs with the yields indicated in Fig. 2. Subsequent 'G'-track sequence analysis of selected mutants within each pool allowed for the identification of all mutant types with the exception of the AAA-COT triple mutant (all six progeny in this pool were the ATA-COT double mutant). Rather than screen additional progeny, the AAA-COT mutant was generated in a separate site-directed mutagenesis experiment by using a specific oligonucleotide as described in the MATERIALS AND METHODS section.

Each mutant COT was produced in recombinant form in *E. coli* and dialyzed crude cell extracts were used to determine the specific activity of each mutant and its K_M value toward carnitine. These values are listed in Table 1. The endogenous acyl-CoA hydrolase activity that is present in *E. coli* prevented an accurate determination of the K_M values of the mutant enzymes toward decanoyl-CoA in the crude cell extracts. However, the latter value and the value of k_{cat} were determined for the AAA-COT triple mutant following its purification to near-homogeneity (see Fig. 3A) by the same procedure used to purify the wild type enzyme (6). These values are listed in Table 2. A Western blot analysis of the crude extracts, shown in Fig. 3, indicates that all of the mutants were present in the extracts in similar amounts, indicating that the specific activity values listed in Table 1 reflect the effects of the mutation(s) on k_{cat} (the assumption being that the K_M value for decanoyl-CoA has not been substantially altered in the single and double mutants, as found for the AAA-COT triple mutant).

The kinetic data obtained for the AAA-COT mutant (Table 2) show that the K_M value for decanoyl-CoA is essentially unaffected by the triple mutation whereas the K_M value towards carnitine is increased by about 10-fold and the value of k_{cat} has been reduced by a factor of about 50. In the case of the single-point mutants, the replacement of serine 473 or threonine 474 with alanine primarily affects the K_M value of the enzyme towards carnitine whereas the replacement of serine 475 with alanine primarily affects the value of k_{cat} (Table 1). The catalytic properties of the double mutants (Table 1) show that the effects of the point mutations are not simply additive but appear to involve compensatory effects, either advantageous or disadvantageous to the efficiency of the enzyme.

DISCUSSION

There has been speculation for some time that the carnitine acyltransferase reaction mechanism may involve the formation of an acyl-enzyme intermediate through the participation of a catalytic asp-his-ser

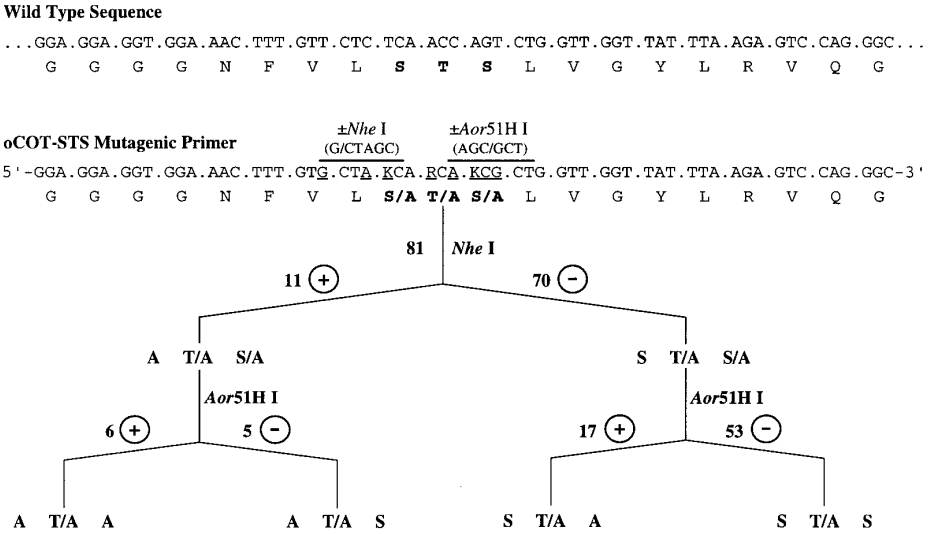

FIG. 2. Site-directed mutagenesis strategy used to generate and to identify mutations at the conserved STS motif in COT. Site-directed mutagenesis of the conserved STS motif that occurs at residues 473–475 in bovine COT was carried out as described in the MATERIALS AND METHODS section. The degenerate mutagenic oligonucleotide oCOT-STS (the underlined bases are mutagenic; K represents a G or T base and R represents an A or G base) was designed to allow for alanine substitutions to occur in all possible combinations at the STS site, such that all single, double and triple mutants (including the wild type sequence with silent mutations in the codons for residues 471, 472, 474 and 475) would be produced. The mutant progeny were initially segregated into four pools of two different mutants per pool based on the presence or absence of novel restriction sites within the plasmid DNA for the enzymes *Nhe* I and/or *Aor*51H I. Cleavage by *Nhe* I indicated that substitution of serine 473 with alanine had occurred whereas failure of *Nhe* I to restrict the DNA indicated that serine was present at this position. A similar analysis with *Aor*51H I allowed the amino acid at position 475 to be identified as either serine or alanine. In order to identify the amino acid at position 474, 'G'-track sequence analysis was carried out on selected mutants from each pool (in fact, since the amino acid at all three positions was decided by the presence or absence of a G base at the first position of the codon, all mutants could be "fingerprinted" by such an analysis). The mutant yields among 81 progeny examined were as indicated in the Figure. The AAA–COT triple mutant was not present among these 81 clones and, rather than screen additional progeny, this mutant was generated with a specific mutagenic oligonucleotide as described in the MATERIALS AND METHODS section. The positive symbol (+) indicates cleavage with the respective restriction enzyme whereas the negative symbol (−) indicates that cleavage did not occur.

triad in a manner similar to that deployed by a variety of esterases, lipases and proteases. However, as described in the Introduction, steady-state kinetic data reported for CAT, COT and CPT-II suggest that each of these enzymes catalyzes acyl-group transfer *via* mechanisms that involve ternary complex formation, supporting the notion that these enzymes are unlikely to make use of a conventional catalytic triad. In addition, Brown *et al.* (5) have pointed out that the Gly-*X*-Ser-*X*-Gly motif (24) that typically identifies the catalytic serine in enzymes operating such a triad is absent from the carnitine acyltransferase sequences.

TABLE 1

Kinetic Constants Determined for the Forward Reactions Catalyzed by a Variety of Recombinant Bovine Liver COT Enzymes Mutated at the Conserved STS Motif

Enzyme	K_M (μM) L-Carnitine	V_m^{app} (u · mg^{-1}) × 10^3	V_m^{app}/K_M (M^{-1} · u · mg^{-1})
STS-COT (Wild Type)	81.9 ± 14.4	109 ± 4	(1.33 ± 0.24) × 10^6
ATS-COT	1,390 ± 380	44.3 ± 6.5	(3.19 ± 0.99) × 10^4
SAS-COT	7,620 ± 530	139 ± 4	(1.82 ± 0.14) × 10^4
STA-COT	51.3 ± 3.3	10.3 ± 0.2	(2.01 ± 0.13) × 10^5
AAS-COT	5,880 ± 1,170	8.77 ± 0.84	(1.49 ± 0.33) × 10^3
ATA-COT	373 ± 120	1.68 ± 0.15	(4.50 ± 1.50) × 10^3
SAA-COT	303 ± 29	9.81 ± 0.27	(3.24 ± 0.32) × 10^4
AAA-COT	794 ± 235	1.38 ± 0.11	(1.74 ± 0.53) × 10^3

Note. Dialyzed crude cell extracts of *E. coli* containing the indicated COT enzymes were prepared as described in the MATERIALS AND METHODS section. In all cases the fixed concentration of decanoyl-CoA was 50 μM. Other assay details were as described in the MATERIALS AND METHODS section. The protein concentrations of the dialyzed crude cell extracts were determined from the absorbance at 280 nm according to $A_{280}^{1\%} = 10$.

TABLE 2

Kinetic Constants Determined for the Forward Reactions Catalyzed by Purified Wild Type and AAA–COT Mutant Forms of Recombinant Bovine Liver COT

Enzyme	Substrate	K_M (μM)	V_m^{app} (u · mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})
Wild Type COT	decanoyl-CoA	1.38 ± 0.53	22.5 ± 2.01	26.6 ± 2.4	(1.93 ± 0.76) × 10^7
	L-carnitine	94.8 ± 5.6	17.0 ± 0.3	20.5 ± 0.3	(2.16 ± 0.13) × 10^5
AAA-COT	decanoyl-CoA	0.206 ± 0.006	0.325 ± 0.008	0.418 ± 0.011	(1.86 ± 0.08) × 10^6
	L-carnitine	992 ± 37	0.379 ± 0.004	0.446 ± 0.005	450 ± 18

Note. The enzymes were purified to near-homogeneity as described previously for Wild Type COT (6). When decanoyl-CoA was the varied substrate, the fixed concentration of L-carnitine was 10 mM. When the concentration of L-carnitine was varied, the fixed concentration of decanoyl-CoA was 50 μM. The close similarity of the kinetic constants determined for recombinant Wild Type COT to those determined for the enzyme isolated directly from beef liver (4,23) indicates that the rapid-equilibrium random-order mechanism followed by the latter enzyme (4) is likely to be obeyed also by the recombinant enzyme. Therefore the values of K_M are listed as true K_M values. The values of k_{cat} were calculated from the values of V_m^{app}($V_m^{app} = V_m/(1 + K_M^B/[B])$, where B is the fixed substrate and V_m equals k_{cat}·[COT]).

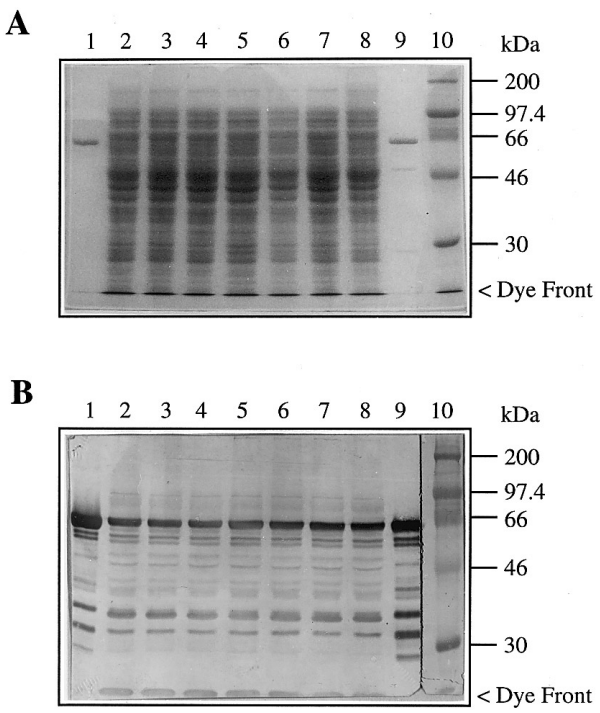

FIG. 3. Detection of mutant COT enzymes in crude extracts of *E. coli* by Western blot analysis. 10% SDS–PAGE slab gel electrophoresis was carried out as described previously (6) on various preparations of COT. Lanes 1 and 9 contained 1 μg of protein, respectively, of purified AAA–COT and Wild Type recombinant COT preparations, whereas Lanes 2–8 contained, respectively, 100 μg of protein from crude extracts of *E. coli* containing recombinant SAA-, ATA-, AAS-, STA-, SAS-, ATS-, and STS–COT (the latter is Wild Type COT whose encoding DNA contains a number of silent mutations compared to that of the natural DNA sequence). Lane 10 contained the molecular weight marker proteins myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). A: The gel was stained for protein. B: The fractionated protein samples were transferred to a nitrocellulose filter as described in the MATERIALS AND METHODS section. Lane 10 was dissected away and stained for protein, whereas the remainder of the filter was probed sequentially with anti-bovine COT polyclonal antibody #501 and an appropriate secondary antibody as described in the MATERIALS AND METHODS section.

An alignment of 11 carnitine acyltransferase sequences indicates that only two serine residues appear to be conserved among all of these enzymes and that both are located within a STS motif that is present also in the choline acetyltransferases (see Fig. 1). The replacement by alanine of all three residues within this motif in COT resulted in an enzyme (AAA-COT) that, in comparison to wild type, had a 10-fold increase in the K_M value toward carnitine and a reduction in k_{cat} of about 50-fold, but with little effect on the K_M value towards decanoyl-CoA (Table 2). While these changes show that the enzyme has been substantially handicapped by the multiple mutation, they demonstrate also that the enzyme retains a considerable k_{cat}/K_M value of 450 M^{-1}s^{-1} for carnitine turnover. Therefore, while it is clear that the residues within the STS motif do have significant roles in COT catalysis, one may conclude that none of the sidechain hydroxyl groups within the conserved STS motif is essential for catalytic activity, thereby eliminating any consideration of these residues as participants in the formation of an acyl-enzyme intermediate. Together with the kinetic data reported by others, these results support the suggestion of Brown *et al.* (5) that the nucleophile in the carnitine acyltransferase reaction mechanism is the deprotonated C3 hydroxyl group of carnitine itself.

The catalytic roles of the individual sidechain hydroxyls within the STS motif may be approximated from consideration of the effects of the point mutations on the kinetic properties of the enzyme (Table 1). The replacement of serine 473 (ATS-COT) or threonine 474 (SAS-COT) with alanine increases the K_M value towards carnitine by factors of 15 and 80, respectively, but with modest effects on k_{cat}. Bovine COT follows a rapid-equilibrium random order mechanism (4) where the substrate K_M values equal their dissociation constants (K_s). Since the mutant enzymes are unlikely to follow a different mechanism, these results suggest that the roles of serine 473 and threonine 474 are in binding carnitine to the enzyme. In contrast, the replacement of serine 475 with alanine (STA-COT) has

a relatively minor effect on the K_M value toward carnitine but reduces k_{cat} by about a factor of 10, suggesting that the role of the sidechain hydroxyl of serine 475 is in transition-state stabilization. Although the K_M value towards the acyl-CoA substrate was not determined for the majority of the enzymes mutated at the STS motif, this parameter was not substantially affected in the AAA-COT triple mutant (Table 2), suggesting that the STS motif does not participate in binding this substrate. Together with the considerations of Brown *et al.* (5) (see above), these results suggest that the hydroxyl groups provided by the side-chains of the residues within the STS motif may contribute to the creation of a hydrogen-bonding network that helps to orient the C3 hydroxyl group of carnitine prior to, and during, catalysis. The conservation of the STS motif in the choline acetyltransferases suggests that these residues fulfill similar roles, *albeit* at the C1 hydroxyl of choline, in that group of enzymes.

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