Specificity of the Interactions between Glu-3, Ser-24, and Gln-30 within the N-Terminal Segment of Rat Liver Mitochondrial Overt Carnitine Palmitoyltransferase (L-CPT I) in Determining the Malonyl-CoA Sensitivity of the Enzyme†

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ABSTRACT: Using deletion mutants of rat liver-type carnitine palmitoyltransferase I (L-CPT I) expressed in *Pichia pastoris*, two contiguous discrete sequences within its N-terminal segment have been shown to be positive (residues 3−18) and negative (19−30) determinants, respectively, of the malonyl-CoA sensitivity of the enzyme. The specific interactions among the three individual residues responsible for these opposing effects within these two regions are here investigated in the context of the full-length protein. The pro-inhibitory effects are due to Glu-3 [Shi et al. (1999) *J. Biol. Chem. 274*, 9421−9426]. We now find that Asp can only partially substitute for Glu-3, whereas the Glu-3Gln mutation has the same effect as the Glu-3Ala mutation. This suggests that a negative charge in this position is essential and that the longer side chain of glutamate is essential for optimal malonyl-CoA sensitivity. Residues within the predicted α-helical 19−30 region responsible for decreasing the sensitivity to malonyl-CoA are shown to be neither the three basic (Arg-22, His-25, and Lys-29) nor the two acidic (Asp-20 and Glu-26) residues, as their mutation to Ala produced only small positive effects on malonyl-CoA sensitivity. The residues responsible were identified as Ser-24 and Gln-30, and their effect was shown to be entirely dependent on the presence of Glu-3. This result reveals that the major sensitization of L-CPT I to malonyl-CoA observed upon deletion of residues 19−30 is not due to a spacer effect with respect to Glu-3 but rather the loss of the two specific residues now identified.

Carnitine palmitoyltransferase I (CPT I) of the mitochondrial outer membrane catalyzes a reaction by which major control is exerted over the rate of fatty acid β-oxidation in all mammalian tissues. As such, regulation of its activity by the potent inhibitor malonyl-CoA (*1*) is important in determining the rate of energy conversion, particularly in oxidative muscles. In mammals, the enzyme exists in two isoforms, the L- (liver) and M- (muscle) isoforms. The L-isoform is present, in addition to hepatocytes, in other sites, notably the pancreatic β-cell (*2*). In addition to its role in energy transformation, CPT I activity is important in determining the effective concentration of long-chain acyl-CoA esters in the cytosolic compartment (*3*). These molecular species are not only highly active in their own right, being involved in the control of gene transcription and membrane trafficking, but also give rise to other molecular species, including diacylglycerols and ceramides, that participate in, and/or interfere with, signal transduction and apoptotic events (*1*). This function is most prominently demonstrated in the pancreatic β-cell, in which diversion of acyl-CoA away from oxidation results in lipoapoptosis (*4*) and in myocytes, where delivery of fatty acyl moieties in excess of the capacity of the tissue to oxidize them results in tissue insulin resistance (*5*).

The kinetic characteristics of L-CPT I differ from those of M-CPT I in several important respects, and particularly in the sensitivity to malonyl-CoA (*6*). Besides the 10-fold difference in the IC_{50} (concentration of inhibitor required to effect 50% inhibition) of the two isoforms, L-CPT I is unique in responding to different physiological states (e.g., starvation and insulin deficiency) by changing its sensitivity to the inhibitor by severalfold (*7*). These changes are thought to amplify the effects of changes in the cytosolic concentration of malonyl-CoA itself that occur in tissues expressing L-CPT I under these conditions (*8*). L-CPT I changes its sensitivity by responding to changes in the lipid composition of the membrane in which it resides (*9*, *10*) and with which it interacts through its two transmembrane domains (*11*). The polytopic membrane topology of the protein results in both a short (46-residue) regulatory N-terminal segment and a large (652-residue) catalytic C-terminal segment being exposed on the same (cytosolic) aspect of the membrane (*11*). Thus, these N- and C-terminal segments can interact, subject to the constraints imposed by the transmembrane segments (*7*). The requirement for the presence of the extreme N terminus for the expression of malonyl-CoA sensitivity was established in early work (*11*) and confirmed by the

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[1] Abbreviations: CPT I, the malonyl-CoA-sensitive carnitine palmitoyltransferase of the mitochondrial outer membrane.

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demonstration that the N-terminal 18 amino acid residues (*12*) and, in particular, Glu-3 (*13*) are essential for the expression of full malonyl-CoA sensitivity by L-CPT I. In subsequent work, we have shown that both N to C segment and inter-TM interactions are important in determining the kinetic properties of L-CPT I (*14*). In addition, using deletion mutants, we found that a short primary sequence within the N-terminal segment has the opposite effect on malonyl-CoA sensitivity to that of deleting Glu-3 (*15*). The region Ile-19−Gln-30 was shown to be a strong negative determinant of this parameter (*15*). We postulated that modulation of the interaction between these two regions by altered membrane−protein interactions, either directly or through their respective opposing effects on the C segment, would give rise to the wide range of sensitivities observed for L-CPT I in liver mitochondria isolated from rats in different metabolic conditions.

To determine whether the effect of the deletion of the 19−30 region was due to either (i) a nonspecific effect owing to altered secondary structure as, for example, would result from a spacer effect with respect to the position of Glu-3 relative to the membrane/rest of the protein or (ii) the removal of specific residues that are important for the precise folding of the protein in response to malonyl-CoA binding, we investigated the effect of point mutations in this predicted α-helical region within the context of the whole protein molecule. We find that whereas replacement of all acidic and basic residues within this region had a relatively modest effect, the specific point mutation of only two amino acid residues, Ser-24 and Gln-30, accounts for the entire effect of the deletion of region 19−30 observed previously (*15*). Their effect is additionally shown to occur in antagonism to the pro-inhibitory effect of Glu-3, rather than independently. We have also determined the charge and size requirements of the side chain at position 3 for the expression of the strong positive effect of Glu-3 on malonyl-CoA sensitivity.

MATERIALS AND METHODS

Sources of materials, methods for transformation, and culturing and preparation of extracts from *Pichia pastoris* and for CPT I assay were as in refs *14*−*16*. All clones were selected with 0.1 mg/mL zeocin.

Generation of Mutants of Rat L-CPT I. The D20A, E26A mutant and the triple mutant R22A, H25A, K29A were generated by replacing the *Spe*I (vector site)−*Bgl*II fragment of pGEM-5Zf containing the 5′ end of L-CPT I with PCR products generated using primer A (ATAGACTA GTTCGAAGATGGCAGAGGCTCACC, which contains an *Spe*I site and a nested *Csp*45I restriction site) in combination with primer B (ATAAGATCTGTTTTAA AGCAGCGTGAGATAATCGTAAAGCAATGCCATCG) or C (AAAAGATCTGTGCCAAAGCTTCTGCTGATAATGCGAGGTCAATGCCAT), respectively. The two *Csp*45I−*Bgl*II fragments were then transferred into rat L-CPT I in pGAPZ B to generate the finished mutants. S24A was made in the same way, except using primers A and D. Primer D (TATAGATCTGTTTTAAAGCTTCATGTGCCAGGCGGAGGT CAAT) introduced a silent *Hin*dIII restriction site. Q30A was generated using primers A and E (TTTTTTGGTCTCCGATCGCTTTGAGGGCTTCGT). Primer E contained a *Bsa*I restriction site, which when cleaved formed a fragment with a *Bgl*II compatible end. The *Spe*I−*Bsa*I cleaved product was then cloned and subcloned into L-CPT I in pGAPZ as above. The double-mutant S24AQ30A was generated using a *Hin*dIII−*Afl*II cleaved PCR product from primers F (CTTCCGATAGAAGCTTTAAAAGCGATCTGCCTGTC) and G (AGATCTTGGTGCTGCGGCTCATTTTGCCGTGTTCTG), which was used to replace the corresponding fragment in the S24A mutant. E3AS24AQ30A was made by replacing the *Csp*45I−*Afl*II fragment of L-CPT I with cleaved PCR products generated using primers H (TCTGTTCGAAGATGGCTGCTGCTCACCAAGCTGTG) and I (CTGCGGCTCATTTTGCCGTGTTCTGCAAACAT) using the S24AQ30A mutant DNA as template. E3D and E3Q were made in the same way, except with wild-type L-CPT I as template, using *Csp*45I−*Afl*II cleaved PCR products generated using primers J (TCTGTTCGAAGATGGCTGATGCTCACCAAGCTGTG) and I and K (TCTGTTCGAAGATGGCTCAAGCTCACCAAGCTGTG) and I, respectively.

Data Analysis. Curve fitting and calculation of kinetic parameters were performed as described previously (*14*, *15*) with the following exceptions: For S24A, Q30A, and S24AQ30A it was noted that even at very high concentrations of malonyl-CoA there was residual low CPT activity. Therefore, in addition to curve-fitting to an equation for simple competitive inhibition (IC_{50} malonyl-CoA (a), Table 1), the percentage inhibition of CPT activity was fitted to the Michaelis−Menten equation. This enabled calculation of maximum inhibition—which in all cases was >85%—and IC_{50} malonyl-CoA [see (b) in Table 1]. For the proteins expressed by the E3AS24AQ30A and E3Q mutants it was observed that incubation of the extracts with low concentrations of malonyl-CoA resulted in an increase in CPT I activity (see below). Therefore, when the apparent IC_{50} value was calculated, the maximal activity was not constrained to that observed in the absence of malonyl-CoA (see Table 1).

RESULTS

Effect of the Replacement of Acidic or Basic Residues within Region 19−*30 of L-CPT I.* Two mutants of CPT I were generated in which either the three basic amino acid residues (Arg-22, His-25, and Lys-29) or both of the acidic residues (Asp-20 and Glu-26) in the 19−30 region were replaced by Ala. Although both of these mutants had lower IC_{50} values (concentrations of malonyl-CoA required for 50% inhibition of CPT activity measured at 35 μM concentration of palmitoyl-CoA) than the wild-type protein, the resultant IC_{50} values (12.9 ± 1.5 and 15.8 ± 1.0 μM, respectively) were still an order of magnitude higher than that previously observed (1.1 ± 0.1 μM) after deletion of the 19−30 region (*15*).

Effect of Ser-24Ala and Gln-30Ala Mutations on L-CPT I Kinetic Parameters. Both Ser-24Ala and Gln-30Ala mutants showed a >10-fold increase in the sensitivity to malonyl-CoA, that is, similar to that shown by the Δ(19−30) mutant in our earlier study (*15*). The IC_{50} value was decreased from 38 ± 2 to 1.88 ± 0.24 and 2.84 ± 0.19 μM for S24A and Q30A, respectively (Table 1). Moreover, these changes in IC_{50} were reflected closely by the decreases in the respective K_i values for malonyl-CoA inhibition of the two mutants (Table 1 and Figure 1). This indicated that the changes in

Table 1: Kinetic Parameters for L-Carnitine, Palmitoyl-CoA, and Malonyl-CoA for Wild-Type and Mutant Forms of Rat Liver CPT I Expressed in *P. pastoris* and Assayed in Cell Extracts[a]

construct	IC_{50} malonyl-CoA (a)	IC_{50} malonyl-CoA (b)	K_I malonyl-CoA	$K_{0.5}$ palmitoyl-CoA	K_m carnitine
wild-type	38 ± 2	N/A	31.0, 41.3	46.0 ± 5.1	153 ± 11
D20AE26A	15.8 ± 1.0	N/A	ND	ND	ND
R22AH25AK29A	12.9 ± 1.5	N/A	ND	ND	ND
S24A	1.9 ± 0.2	1.24 ± 0.1	1.74, 1.44	86.9 ± 7.3	158 ± 4
Q30A	2.8 ± 0.2	1.77 ± 0.1	1.51, 1.40	87.9 ± 8.0	162 ± 4
S24AQ30A	1.1 ± 0.1	0.67 ± 0.06	1.08, 1.30	72.7 ± 2.1	164 ± 5
E3D	79.0 ± 7.2	N/A	ND	ND	ND
E3Q*	205 ± 9	N/A	ND	ND	ND
E3AS24AQ30A*	172 ± 21	N/A	ND	ND	ND

[a] For all parameters, values (μM) are means (± SE) of at least three independent preparations, except for K_i determination ($n = 2$). See Materials and Methods for details of graphical analyses. For S24A, Q30A, and S24AQ30A, IC_{50} values for malonyl-CoA were calculated by two methods (a, b), one of which (b) took into account the incomplete inhibition of the enzyme activity even at very high malonyl-CoA concentrations (see Materials and Methods). Asterisks indicate that only an apparent IC_{50} value could be calculated, due to the increase in CPT activity at low concentrations of malonyl-CoA (see Materials and Methods, Figure 2, and text). The data for the wild-type protein are taken from ref *15*. ND, not determined; N/A, not applicable.

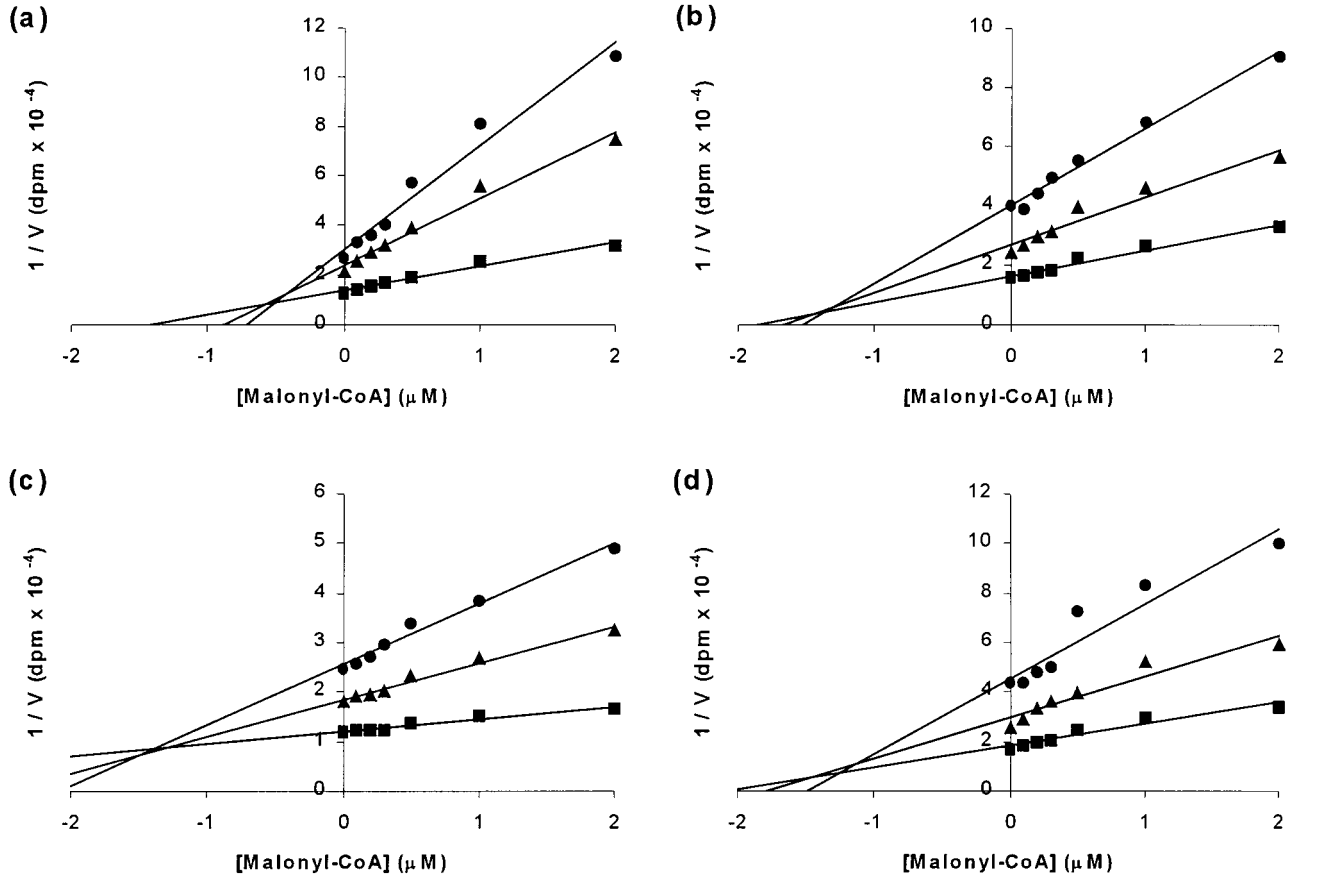

FIGURE 1: Dixon plots for the inhibition of CPT I mutant activities by malonyl-CoA, assayed in cell-free extracts of *P. pastoris* in which they were expressed. Constructs were expressed in *P. pastoris* as follows: (a) Δ(19−30), (b) S24A, (c) Q30A, and (d) S24AQ30A. CPT activity was measured at a range of malonyl-CoA concentrations in the presence of 500 μM carnitine and 20 μM (●), 35 μM (▲), and 70 μM (■) palmitoyl-CoA, respectively. Plots representative of data obtained with two separate preparations, which gave very similar results, are shown.

sensitivity were not due to changes in the mechanism of inhibition of the enzyme but due to a genuine increase in affinity for malonyl-CoA. The combined effects of the S24A and Q30A mutations were tested in the double-mutant S24AQ30A. The malonyl-CoA sensitivity of this mutant was even higher than that of the individual mutations, taking the IC_{50} and K_i values of the mutant L-CPT I to values that were identical to those achieved by the Δ(19−30) mutation (see Table 1 and ref *15*).

In addition to the effectiveness of the Ser-24Ala and Gln-30Ala mutations in mimicking the effect of the Δ(19−30) deletion on malonyl-CoA sensitivity, the two individual mutations and the double mutant (S24AQ30A) also mimicked the effect of the 19−30 deletion in their effect on the apparent K_m for palmitoyl-CoA. In all instances this was increased by ~2-fold (Table 1 and ref *15*).

When compared to the wild-type mutant, there were no major changes in the K_m for carnitine of any of the point

mutants. This was in contrast to the observations on the Δ-(19−30) and Δ(19−46) deletion mutants studied previously. For those mutants, a small (1.5-fold) but significant increase in this parameter was observed (*15*). This difference between the deletion and point mutants suggests that the effect on carnitine kinetics observed for the deletion mutants was due to large nonspecific perturbations caused by the deletions themselves.

Interaction between the Effects of Glu-3 and of Ser-24 and Gln-30. We have previously shown that the Glu-3Ala mutation can over-ride the effects of the 19−30 deletion (*15*). Therefore, we tested whether Glu-3Ala can also over-ride the combined effects of S24A and Q30A mutations. Table 1 shows that the S24AQ30A double mutations cannot exert their positive effects on malonyl-CoA sensitivity in the absence of Glu-3.

Specificity of Charge and Side-Chain Size Requirements at Position 3. In view of the importance of the presence of Glu-3 for malonyl-CoA sensitivity, both in the wild-type enzyme (*13*) and in the S24AQ30A double mutant, we decided to examine the role of this residue further. The effects of the replacement of Glu-3 in rat L-CPT I with an Asp residue are shown in Table 1. It can be seen that the Glu-3Asp mutant has a malonyl-CoA sensitivity that is of the same order of magnitude as that of the wild-type; its IC_{50} for malonyl-CoA was 2-fold higher. This suggests that aspartate, which has a shorter side chain, can only partially substitute for glutamate at this position. The inability of a glutamine residue, in the mutant Glu-3Gln, to mimic the effect of a glutamic acid residue at this position (Table 1) indicates that a negative charge at position 3 is essential for the malonyl-CoA sensitivity of L-CPT I but that the carbonyl group per se is not responsible for the effect.

Interactions between Malonyl-CoA and Palmitoyl-CoA Kinetics of Glu-3Ala Mutants. In the present study, we noticed that in assays of E3AS24AQ30A activity, addition of low concentrations of malonyl-CoA increased the CPT activity of the expressed protein (Figure 2). This phenomenon indicates that hyperbolic inhibition occurs for this mutant as observed for all other E3A-containing mutants (*15*) and that the increase in the activity of the protein owing to this V_{max} effect is sufficient to overcome the small inhibitory effect of malonyl-CoA, at low concentrations of the inhibitor (≤10 μM). Under our assay conditions, the highest activity observed was 113 ± 1% of that measured in the absence of malonyl-CoA. Increasing the concentration of malonyl-CoA above 10 μM overcame this V_{max}-related effect, and net inhibition was observed (Figure 2).

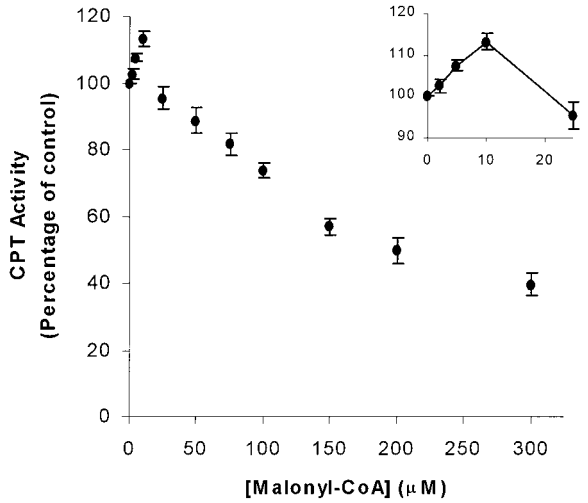

FIGURE 2: Effects of increasing concentrations of malonyl-CoA on the activity of the triple mutant E3AS24AQ30A L-CPT I. Cell-free extracts of *P. pastoris* expressing E3AS24AQ30A were prepared, and CPT activity was measured. Aliquots (25 μL) of extract were measured in a final volume of 1 mL for 4 min at 30 °C, using 35 μM palmitoyl-CoA and 500 μM carnitine. Values are means (± SE) from four separate preparations, each assayed in duplicate. Where error bars are not visible, they lie within the symbol. (Inset) The CPT activity at ≤25 μM malonyl-CoA is displayed on an expanded scale to show more clearly the increase in CPT activity observed at these concentrations.

DISCUSSION

Roles of Glu-3, Ser-24, and Gln-30. The first indication that the predicted α-helical sequence containing Ser-24 and Gln-30 is a negative determinant of malonyl-CoA sensitivity of L-CPT I emerged from our previous studies (*15*) in which the IC_{50} values of N-terminally truncated Δ(3−18) L-CPT I were compared with those of the catalytic core Δ(1−82). The latter had an IC_{50} for malonyl-CoA that was severalfold lower than that of the truncated mutant, indicating that there is a negative determinant of malonyl-CA sensitivity between residue 19 and the first transmembrane segment (*15*). Deletion of region 19−30 proved this to be the case, as the resulting mutant had IC_{50} and K_i values that were >50-fold lower than those of the parental L-CPT I. Two possibilities exist to explain this effect, namely, (i) that this 19−30 region acts as a molecular spacer, keeping the extreme N terminus [which had previously been shown to be essential for high-affinity inhibition by malonyl-CoA (*15*)] at a critical distance from crucial residues within the N or C termini and/or the membrane, or (ii) that individual residues within this region act by altering the affinity of the protein for malonyl-CoA. Therefore, we elected to test the latter possibility directly by expressing mutants in which specific residues, or groups thereof, were mutated to Ala within the context of full-length CPT I. To determine the functionally important residues in the 19−30 region, we initially mutated either all three basic residues (which represented potential partners for interaction with the negative charge of Glu-3) or the two acidic residues. The two resulting mutants had slightly lower IC_{50} values for malonyl-CoA than the wild-type protein (Table 1), but the effect was modest compared to that observed for the Δ(19−30) mutant (*15*). Therefore, it is likely that such modest changes result indirectly, for example, from small conformational changes that alter the native interactions.

It was reasoned that, of the remaining residues (Ile, Leu, Ser, Ala, and Gln) within the 19−30 region, Ser-24 and Gln-30 were the most likely candidate residues for affecting malonyl-CoA sensitivity through interactions involving Glu-3, as they are the only ones not having exclusively aliphatic side chains. Mutation of either of these two amino acids to alanine resulted in an ∼10−20-fold lowering of the IC_{50} and K_i values for malonyl-CoA of the expressed proteins (Table 1). Moreover, the double mutant (S24AQ30A) had even lower values for these parameters and fully mimicked the effects of the Δ(19−30) deletion (*15*) with respect to malonyl-CoA inhibition (Table 1).

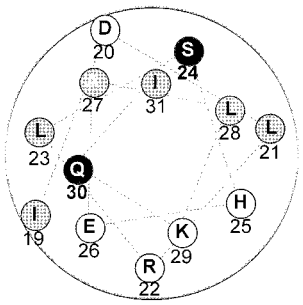

FIGURE 3: Helical wheel prediction for residues 19−31 of L-CPT I.

The possible steric relationships of these two residues with respect to each other and with the rest of the residues that make up this putatively α-helical stretch of the N-terminal segment of L-CPT I are shown in Figure 3. The putative α-helical structure stretching from residue 23 up to, and including, Gln-30 is remarkable for its amphipathic nature, having on one side three positively charged residues (to the extent that His-25 is protonated) and on the opposite side exclusively hydrophobic residues, apart from Ser-24 and Gln-30 themselves.

Mechanism of Action of Glu-3. The specific importance of Glu-3 in the expression of malonyl-CoA-sensitivity by L-CPT I was first demonstrated in ref *13*. The present data indicate that the negative charge associated with Glu-3 is essential for retention of high-affinity malonyl-CoA inhibition and that its effect can be mimicked only partially by replacement with an Asp residue and not at all by substitution with Gln. These observations suggest that the size of the side chain is also important and that Glu-3 needs to interact intramolecularly across a critical distance, possibly between the N-terminal and catalytic C egments.

Previously, we showed that the Δ(19−30) mutation did not alter the mechanism of malonyl-CoA inhibition with respect to palmitoyl-CoA, because the increase in effectiveness of malonyl-CoA as an inhibitor coincided with a genuine increase in the affinity of the protein for the inhibitor (*15*). This is mimicked by all three of the mutants involving Ser-24 and/or Gln-30 in the present study (Table 1). By contrast, the large increase in IC_{50} displayed by the E3A mutant was not accompanied by any change in K_i for malonyl-CoA (*15*). In addition, it was not associated with any major change in the affinity for palmitoyl-CoA. Moreover, in the E3A mutant, the apparent $K_{0.5}$ for palmitoyl-CoA was increased, indicating that the increase in IC_{50} for malonyl-CoA could not have resulted from an increased affinity for palmitoyl-CoA. Therefore, we suggest that the increase in IC_{50} for the E3A mutant is largely due to the concomitant change in mechanism of interaction between malonyl-CoA, palmitoyl-CoA, and their respective binding sites. That a different type of inhibition by malonyl-CoA [hyperbolic inhibition (*17*)] is induced generally in E3A-containing mutants of L-CPT I is fully supported in the present study by the similar, but more pronounced, kinetic behavior of the triple-mutant E3AS24AQ30A. Thus, like E3A, the E3AS24AQ30A mutant showed a significant increase in CPT activity at low malonyl-CoA concentrations (≤10 μM) indicating that, as predicted by hyperbolic inhibition, the protein becomes a better substrate for palmitoyl-CoA when it binds malonyl-CoA. In this type of inhibition, the binding of a small inhibitor molecule that shares molecular structural characteristics of a substrate favors the binding of a larger substrate molecule through the induction of increased ordering of the binding site (*17*). At such low malonyl-CoA concentrations this effect on palmitoyl-CoA kinetics is strong enough to overcome the weak malonyl-CoA-mediated inhibition and to result in a net increase in CPT activity, even at the suboptimal concentration of palmitoyl-CoA used in the assay. It is inferred that as the concentration of malonyl-CoA is increased (>10 μM) the greater inhibitory effect of malonyl-CoA masks the effect due to elevated V_{max} (Figure 2). Interestingly, precisely this type of behavior has been described previously for L-CPT I solubilized from rat liver mitochondria and resedimented with poly(ethylene glycol) (*18*). We suggest that those observations were due to N-terminal truncation of the protein, with consequent loss of Glu-3 (cf. ref *11*) by protease action during these in vitro manipulations.

The identification of two specific residues, Ser-24 and Gln-30, as negative determinants of malonyl-CoA sensitivity of L-CPT I is highly significant, as the observation raises the possibility that these residues might interact not only with Glu-3 within the N-terminal segment but also with the active site of the protein (*19*) and thus could be targeted pharmacologically for modulation of the malonyl-CoA sensitivity of CPT I in vivo. More fundamentally, the extent to which this (these) interaction(s) can occur is expected to be influenced by the proximity of the transmembrane segments, owing to the membrane topology of the CPT I molecule (*20*) and their mutual interactions, thus contributing to the responsiveness of the malonyl-CoA sensitivity of L-CPT I to the physical properties of the membrane in which it resides (*7*).

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