Redesign of Choline Acetyltransferase Specificity by Protein Engineering*

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Since the development of site-directed mutagenesis techniques over 15 years ago (Zoller, M. J., and Smith, M. (1982) Nucleic Acids Res. 10, 6487–6500), it has been a goal of protein engineering to utilize the procedure to redesign existing enzyme structures to produce proteins with altered or novel catalytic properties. To date, however, the more successful achievements have relied exclusively on the availability of three-dimensional protein structure maps to direct the redesign strategies. Presently, such maps are unavailable for choline acetyltransferase and carnitine acetyltransferase, enzymes that catalyze the reversible transfer of an acetyl group from acetyl-CoA to choline and L-carnitine, respectively. A more empirical approach, based on cross-referencing substrate structure comparisons with protein alignment data, was used to redesign choline acetyltransferase to accommodate L-carnitine as an acceptor of the acetyl group. A mutant choline acetyltransferase that incorporates four amino acid substitutions from wild type, shows a substantial increase in catalytic efficiency ($k_{cat}/K_m$) toward L-carnitine (1,620-fold) and shifts the catalytic discrimination between choline and L-carnitine by $>390,000$ in favor of the latter substrate. These dramatic alterations in catalytic function demonstrate that significant success in protein redesign can be achieved in the absence of three-dimensional protein structure data.

Carnitine acetyltransferase (CAT)† (EC 2.3.1.7) catalyzes the reversible transfer of an acetyl group between acetyl-CoA and L-carnitine (hereafter carnitine). Based partly on the crystal structures of the carnitine and acylcarnitine zwitterions, Gandour et al. (1) have proposed a chemical and topographic mechanism for CAT catalysis in which acyl group transfer proceeds through an addition-elimination reaction via a tetrahedral intermediate. In this scheme, the negatively charged carboxylate and positively charged trimethylammonium groups of carnitine serve to anchor the substrate to the enzyme. These proposals have been elaborated (2) to suggest that a charge-relay system involving an Asp-His couple on the enzyme serves to extract a proton from the C3 hydroxyl group of carnitine that allows for nucleophilic attack of the resulting oxanion on the carbonyl of the acyl-CoA thioester. This model of CAT catalysis is supported by kinetic (3) and site-directed mutagenesis experiments (4) that tend to exclude a modified enzyme intermediate from the reaction pathway and by additional site-directed mutagenesis experiments that demonstrate essential catalytic roles for histidine and aspartate residues (2), and a role for an arginine residue (5) in the formation of a salt bridge with the carbonyl group of carnitine.

Choline acetyltransferase (ChAT) (EC 2.3.1.6) catalyzes a similar reaction to CAT, with the exception that the acetyl group from acetyl-CoA is transferred to choline rather than carnitine. Carnitine differs from choline by having a carboxymethyl group replace a hydrogen at C1 of the latter compound. Relative to the trimethylammonium group of the substrate, acyl group esterification occurs at the same hydroxyl moiety in both enzyme reactions, on C1 in choline and C3 in carnitine. Like CAT (3), ChAT also follows a random-order ternary complex mechanism (6, 7) and also has an essential histidine residue that is believed to act as the general acid/base catalyst (8). Thus, it seems likely that both enzymes follow similar chemical reaction mechanisms. Because the carboxymethyl group of carnitine may be involved strictly in binding (1, 5), it is expected that CAT possesses a suitably constructed, positively charged pocket with which to accommodate and neutralize this group, and that this structure is absent in ChAT. Thus, the engineering of such a structure into ChAT might allow this enzyme to catalyze the acylation of both carnitine and choline.

Although the CDNA and deduced protein sequences of 13 carnitine acetyltransferases and 5 ChATs have been reported (see Fig. 1 for references), a three-dimensional structure has not yet been described for any of these enzymes. It is a generally held belief that such structures are an essential requirement for any meaningful attempts at protein redesign (e.g. see Ref. 9). In the present study, however, this is shown not to be the case. By combining the information from substrate structure comparisons and protein alignment data, four amino acid residues in the primary sequence of ChAT were targeted for replacement. A modified ChAT enzyme, ChAT-R/TET, that contains these four substitutions shows a greater than three orders of magnitude increase in catalytic efficiency ($k_{cat}/K_m$) for the acetylation of carnitine yet retains substantial choline acetyltransferase activity.

EXPERIMENTAL PROCEDURES

**Materials—**

L-Carnitine·HCl and choline·Cl were obtained from Sigma. CM-Sepharose CL-6B was obtained from Amersham Pharmacia Biotech, and synthetic oligonucleotides were purchased from Operon.

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The complete rat brain ChAT cDNA of 2337 bp (supplied on vector pSPT18-rChAT-1; Ref. 10) was a kind gift from Drs. L. Houhou and J. Cronin, manuscript in preparation). With the exception of the initial transformation into Escherichia coli 71-18 mutS (CLONTECH) during the site-directed mutagenesis protocol, all molecular biology experiments in this paper utilized E. coli DH5α F′IQ (Life Technologies, Inc.) as host.

Production of Recombinant Rat ChAT—A restriction site for NdeI encompassing the translational start codon and a HindIII site downstream of the termination codon were introduced into the cDNA encoding rat ChAT by using the polymerase chain reaction with plasmid pSPT18-rChAT-1 (10) as template and oligonucleotides oChAT5 (5′-GGGACGGAGGG-GGCCATGATGCCCTGGA/AAAGGGTC- C-3′) and oChAT3 (5′-CTGAGCTCTGAGCATGCAAGGCCTGCATTG- GCCGGTATGCTTGAACATTG-3′) as amplification primers (the underlined bases are those that differ from the target template). The amplified fragment was digested with NotI (sites present in the primers only) and cloned between the NotI and XhoI sites of pBluescript KS + (Stratagene) to generate the intermediate vector pKS-ChAT. The sequences of the flanking regions of the amplified ChAT structural gene, i.e., lying upstream of the SpHl I-site and downstream of the Nco I-site, were sequenced to assess the integrity of the polymerase chain reaction process. An erroneous base within a subpopulation of the oChAT5 primer required replacement of the NotI-Sphl fragment in pKS-ChAT with the equivalent fragment obtained from the polymerase chain reaction that proved to be the correct sequence. The central 1.542-bp Sphl/NotI fragment within pKS-ChAT was then replaced with the Sphl/NotI fragment from the ChAT cDNA (obtained from pSPT18-rChAT-1) to generate pKS-ChAT.

The rat ChAT structural gene was then assembled between the NdeI and HindIII sites of the E. coli protein expression vector pLENTY by simultaneous ligation of the 737-bp NdeI fragment from pKS-rChAT* to generate pLENTY-rChAT (this was dialyzed against 4 liters of 10 mM KHPO4-KOH buffer, pH 7.0, containing 1 mM EDTA (free acid) (PE buffer) and 0.2 mM dithiothreitol for 4 h).

The dialyzed material was passed through a column (3.2 cm diameter by 15 cm height) of CM-Sepharose FF (Amersham) that had been equilibrated with PE buffer at 1 ml/min, and the column was washed overnight with about 700 ml of PE buffer. ChAT was eluted by applying a linear gradient of 0–0.75 mM KC1 (300 ml total volume) in PE buffer. A conservative pool of the peak fractions of ChAT activity was concentrated to approximately 5 ml by using an Amicon PM-10 membrane and dialyzed overnight against 1 liter of 20 mM KHPO4- KOH buffer, pH 7.5, containing 1 mM EDTA (free acid), 1 mM dithiothreitol, and 50% (v/v) glycerol. The enzyme was stored at −20 °C and was dialyzed against 20 mM KHPO4-KOH buffer, pH 7.5, prior to the determination of kinetic parameters.

Recombinant soluble ChAT was produced by using an extinction coefficient at 280 nm of 8.63 × 104 M−1 cm−1 that was calculated based on its tryptophan and tyrosine content (12) (equivalent to ε280 = 9.43). SDS-polyacrylamide gel electrophoresis was carried out as described previously (5).

Measurement of Enzyme Activity and Treatment of Kinetic Data—During the purification, ChAT activity was determined routinely at 30 °C in 20 mM KHPO4-KOH buffer, pH 7.4, and in the presence of 50 mM acetyl-CoA and 1 mM choline in a total volume of 0.1 ml. The net decrease in the absorbance at 232 nm, due to the hydrolysis of acetyl-CoA, was used to determine ChAT activity by using an extinction coefficient of 4.440 μM−1 cm−1 (13). The activity of ChAT in crude extracts of E. coli was corrected for the presence of an acyl-CoA hydrolase activity by measuring the rate of acetyl-CoA hydrolysis in the absence of choline. The mutant ChAT enzymes were assayed in a similar manner except that 10 mM choline was present in the latter substrate. The activity was measured out by using a Uvikon 810 spectrophotometer (Kontron Instruments).

The steady-state kinetic parameters for the various purified derivatives of ChAT listed in Table I were determined under conditions similar to those described above, but with the exceptions that the assay mixtures also contained 0.2 mM KC1 and the concentrations of both acetyl-CoA and the acceptor substrate, either choline or carnitine, were each varied as required. Carnitine was neutralized with KOH before use. When the assay concentration of choline (as choline chloride) or carnitine (as carnitine hydrochloride) exceeded 10 mM, the KC1 concentration was reduced by an equivalent amount to counteract fluctuations in ionic strength.

ChAT follows a random-order sequential reaction mechanism (6, 7), and thus, each experimental data set was fitted to the equation: 

\[
\frac{v}{K_{cat}} = \frac{[E][A][B]}{K_{M}^{A} + [A][B]} + \frac{[E][A][B]}{K_{M}^{A} + [A][B]}
\]

where v is the measured rate and E, A, and B represent, respectively, enzyme, acetyl-CoA, and choline or carnitine, as appropriate. K_{M}^{A} and K_{M}^{B} are the Michaelis constants and K_{cat} is the dissociation constant for acetyl-CoA. The dissociation constant of acetyl-CoA to carnitine (K_{c}) was calculated from the relationship: 

\[
K_{c} = K_{M}^{B} / K_{cat}
\]

The K_{c} values toward carnitine displayed by the wild type, ChAT-R, and ChAT-TET enzymes exceeded the practical concentration range of this substrate (100 mM). Therefore, the k_{cat}/K_{c} values of these enzymes toward carnitine were determined in the presence of 100 μM acetyl-CoA, which should be sufficient to saturate the enzymes with regard to this latter substrate. The k_{cat}/K_{c} values were calculated by linear least-
Redesign of Choline Acetyltransferase Specificity

As described in the introduction to the text, the engineering of ChAT to utilize carnitine, in addition to its natural substrate choline, as an effective acceptor of the acetyl group from acetyl-CoA might be achieved by introducing a positively charged side chain into the enzyme with which to accommodate the additional negatively charged carboxymethyl group. Because it is expected that the amino acid residues that contribute to the formation of such a structure are already present in the carnitine acyltransferases, it was considered that these residues might be identified from protein alignment data as conserved substitutions between the choline and carnitine acyltransferase families. Specifically, such substitutions were expected to include an additional basic residue in the carnitine acyltransferases, coupled with a reduction of about 60 Å³ in the side-chain volume of one or more amino acid residues.

An alignment of the various choline and carnitine acyltransferases sequences showed that 30 amino acid residues are conserved identically among all proteins (data not shown). When the conservation of residues within each group was compared with the other, a number of distinct differences were identified, as illustrated in Fig. 1. First, Asn-514 in rat ChAT, conserved in four out of five ChAT sequences, is replaced conservatively by Glu in the majority of carnitine acyltransferases. Second, Val-459 and Asn-461 in rat ChAT, the former conserved in all ChAT sequences and the latter in four out of five ChAT sequences, are each replaced exclusively in the carnitine acyltransferases by Thr. In addition, the ionic charge required to interact with, and neutralize, the carboxyl group of carnitine. Secondary structure prediction analyses (17) of these enzymes (data not shown) suggests that each of these tripeptide sequences actually interact with their respective substrates, since recent protein engineering experiments (18, 19) have demonstrated that critical contributions to enzyme specificity and catalytic efficiency may be provided by loop structures that do not directly contact the substrate. Additional conserved differences between the two enzyme families that might contribute to their observed substrate preferences were not readily apparent, and it was felt to providing an alternative spatial arrangement of hydrogen bonding donors/acceptors, these substitutions create a side-chain volume decrease of approximately 33 Å³ (16) in the carnitine acyltransferases. An Asp residue is found to occur between these two amino acids in all ChAT sequences, but is replaced conservatively by Gla in the majority of carnitine acyltransferases, although some CAT sequences retain Asp.

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Thus, with the intention of producing a dual-specific choline/carnitine acetyltransferase or a ChAT that is selective for carnitine, a variety of engineered derivatives of ChAT were produced. These included ChAT-R (Asn-514 replaced with Arg), ChAT-TET (the VDN tripeptide at residues 459–461 replaced with Glu), and ChAT-R/TET (the VDN tripeptide at residues 459–461 replaced with Glu, the Arg at the equivalent location in the carnitine acyltransferases forms a salt bridge with the carboxyl group of carnitine).

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ciency toward choline that is maximal in the ChAT-R/TET quadruple mutant with a reduction of 245-fold from wild type. Although the VDN → TET replacement elevates $k_{\text{cat}}$ by some 3 to 4-fold for choline turnover, the reduction in the catalytic efficiency of the mutant enzymes toward this substrate may be accounted for primarily by an increase in the $K_m$ value. In each mutant, the increase in the $K_m$ value toward choline is mirrored by a similar increase in its dissociation constant ($K_d$), indicating that the loss in catalytic efficiency primarily reflects an impairment in substrate affinity.

In each enzyme derivative, the dissociation constant for acetyl-CoA is similar to that determined for the wild type enzyme, indicating that the mutations do not affect the affinity for the acyl group donor. Thus the modifications appear to have specific, and profound, effects on the binding and/or turnover of the acceptor of the acetyl group. In the case of the ChAT-R/TET enzyme, the net effect of these changes is a shift in the catalytic discrimination between choline and carnitine by a factor of $\geq 390,000$ in favor of the latter compound, with the result that this enzyme acetylates both substrates with rather similar catalytic efficiencies (2.5-fold preference for choline; Table I). Although the amino acid replacements introduced into the ChAT-R/TET enzyme lead to the acquisition of substantial carnitine acetyltransferase activity ($k_{\text{cat}}/K_m = 4.69 \times 10^5 \text{M}^{-1} \text{s}^{-1}$), the enzyme remains quite inferior to native CAT (for example, the data reported for human liver CAT in Ref. 20 suggest an approximate $k_{\text{cat}}/K_m$ ratio of $8.8 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ for carnitine turnover). Thus, while the residues targeted for replacement in the present study contribute substantially to the different substrate preferences displayed by the choline and carnitine acetyltransferase families, it is clear that additional more subtle differences must exist between the two families that account for the remaining differences in the catalytic efficiencies displayed toward their respective natural substrates. Nevertheless, unlike the carnitine acetyltransferases that have little ability to acetylate carnitine (carnitine octanoyltransferase acetylates choline with a $k_{\text{cat}}/K_m$ ratio of $0.212 \text{M}^{-1} \text{s}^{-1}$; Ref. 5), the Chat-R/TET enzyme retains substantial choline acetyltransferase activity ($k_{\text{cat}}/K_m = 1.18 \times 10^5 \text{M}^{-1} \text{s}^{-1}$). Thus, the ChAT-R/TET enzyme represents a novel bifunctional carnitine/choline acetyltransferase.

The improvements engineered into the ChAT-R/TET enzyme for carnitine turnover compare very favorably with the more successful improvements in catalytic efficiency reported in previous redesigns of multisubstrate enzymes, some of which are listed in Table II, in which high resolution three-dimensional protein structure maps were utilized as “blueprints” for the redesign strategies. In the case of multisubstrate enzymes, it has been pointed out (21) that realistic measures of protein-engineered improvements in catalytic efficiency should take into account the properties of the redesigned enzyme with regard to the overall catalytic reaction, i.e. when $k_{\text{cat}}$ and the values of $K_m$ for each substrate are considered as a whole. For the ChAT-R/TET enzyme this “overall catalytic efficiency” (21) is defined as $k_{\text{cat}}/K_m \cdot \rho$, where $\rho$ is the ratio of the number of catalytic turnovers per second to the number of moles of substrate in the reaction mixture, and equals $1.65 \times 10^8 \text{M}^{-2} \text{s}^{-1}$. When compared with the value for wild type, $1.44 \times 10^5 \text{M}^{-2} \text{s}^{-1}$ ($k_{\text{cat}}/K_m$ for carnitine divided by the apparent $K_m$ for acetyl-CoA), the overall gain in catalytic efficiency for acetylation of carnitine is 1,150-fold (this gain may, in fact, be significantly greater since, by comparison with the data obtained for those reactions in which the true $K_m$ value for acetyl-CoA was determined, the true $K_m$ value for acetyl-CoA during turnover of carnitine by the wild type enzyme may well be higher). In Table II, only one of the previous studies with multisubstrate enzymes reported the effects of the redesign on each substrate in the reaction, and thus, the “overall catalytic efficiency” cannot be determined for the majority of studies. Therefore, the relative success of the present redesign may be more profound.

Acknowledgments—I am indebted to Professor Thomas P. Singer for his support of this project. I thank Drs. L. Houhou and J. Mallet (CNRS, Paris, France) for the gift of plasmid pSPT18-rChAT-1.

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