Identification of an Active Site Arginine in Rat Choline Acetyltransferase by Alanine Scanning Mutagenesis*

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Kinetic as well as chemical modification studies have implicated the presence of an active site arginine in choline acetyltransferase, whose function is to stabilize coenzyme binding by interacting with the 3'-phosphate of the coenzyme A substrate. In order to identify this residue, seven conserved arginines in rat choline acetyltransferase were converted to alanine by site-directed mutagenesis, and the properties of these mutants were compared with the wild type enzyme. Substitution of arginine 452 with alanine resulted in a 7–12-fold increase in the $K_m$ for both CoA and acetylcholine as well as in the $k_{cat}$ with little change in the $K_m$ for dephospho-CoA. Product inhibition studies showed choline to be a competitive inhibitor with respect to acetylcholine, indicating that arginine 452 participates in coenzyme A binding. These findings are consistent with Arg-452 mutations increasing the rate constant, $k_{cat}$, for dissociation of the coenzyme from the enzyme. Direct evidence that arginine 452 is involved in coenzyme A binding was obtained by showing a 5–10-fold decrease in affinity of the R452A mutant for coenzyme A as determined by the ability to protect against phenylglyoxal inactivation as well as thermal inactivation.

The enzyme choline acetyltransferase (ChAT; EC 2.3.1.6) catalyzes the transfer of the acyl group from acetyl-CoA to choline resulting in the formation of the neurotransmitter acetylcholine. Mechanistic studies on the enzyme suggest a concerted reaction in contrast to similar enzymes that transfer acyl groups via an acyl enzyme intermediate. The kinetic mechanism for the enzyme approximates a Theorell-Chance mechanism (1), although by using isotope exchange at equilibrium a random component in the reaction has been detected (2). Chemical modification studies have implicated histidine, cysteine, and arginine as active site residues. Thus inactivation studies with dithiobis-4-nitro-2-carboxylate led to the proposal that the enzyme contains an active site histidine (3–7); however, modification of this residue by methylation showed it is not essential for catalysis (8). Similarly, an active site histidine was implicated by inactivation studies with diethylpyrocarbonate while an active site arginine was implicated by inactivation studies with phenylglyoxal (7, 9). It has been suggested that the active site histidine serves as a general acid/base catalyst (10), while the active site arginine is postulated to be involved in binding interactions with the 3'-phosphate of the substrate CoA (9).

With the availability of cDNA clones for ChAT from Drosophila melanogaster (11), porcine spinal cord (12), rat brain (13, 14), mouse brain (14), and Caenorhabditis elegans (15) it has now become possible to use site-directed mutagenesis to identify these active site residues and to study their function. We have previously used site-directed mutagenesis to analyze the functionality of three conserved histidines in Drosophila ChAT and have shown that one of these residues, His-426, is essential for catalysis (16). We have now used a similar approach to search for the active site arginine thought to be involved in coenzyme A binding. Seven conserved arginines were individually changed to alanine and the resultant mutants characterized. The properties of mutant enzymes containing substitutions at arginine 452 are consistent with this arginine serving as an active site residue.

EXPERIMENTAL PROCEDURES

Materials—Citrate synthase and malate dehydrogenase, both from pig heart, were obtained from Sigma. Ni-nitrosoacetate-agarose was obtained from Qiagen, while Immobilon-P nylon membranes were obtained from Millipore Corp. A rabbit anti-human ChAT antiserum was obtained from Bio-Rad.

Expression and Purification of Recombinant ChAT—Escherichia coli SG12036 expressing recombinant rat ChAT was grown at 22 °C for 4 days in LB media. Cells were harvested by centrifugation and frozen at −80 °C until further use. Enzyme purification was achieved as described previously (17). Expression and purification of the recombinant enzyme on a column of Ni-nitrosoacetate-agarose equilibrated with the extraction buffer. The column is washed batchwise with buffer containing increasing concentrations of NaCl from 0 to 2 M and lastly with buffer containing 5 mM imidazole, pH 7.4. The enzyme, eluted with an imidazole gradient from 5 to 150 mM, was applied to a column of blue-agarose (Amicon), previously equilibrated with 20 mM sodium phosphate buffer, pH 7.6. After washing the column with equilibration buffer, the enzyme was eluted with a linear salt gradient from 0 to 1 M and concentrated/dialyzed in a Centricon 30 concentrator (Amicon). The purified enzyme was either used immediately or stored at −80 °C in 20 mM sodium phosphate buffer, pH 7.6, containing 40% glycerol.

Assay of ChAT Activity—Enzymatic activity was determined by the fluorometric assay of Hersh et al. (19). This assay involves coupling the reverse ChAT reaction (CoA + AcCh → AcCoA + Ch) to citrate synthase and malate dehydrogenase. Assays conducted under high salt conditions showed an approximately 2-fold increase in activity compared to those conducted under low salt conditions.

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‡ The abbreviations used are: ChAT, choline acetyltransferase; Ch, choline; PAGE, polyacrylamide gel electrophoresis.
conserved arginines in rat choline acetyltransferase and their conversion to alanine

| Position in sequence | Amino acid sequence | Original codon | Mutant codon(s) |
|----------------------|--------------------|---------------|----------------|
| 99                   | MYNSRLALPVNS       | CGC           | GCG           |
| 250                  | GLTSGBNSEMA        | AGG           | GCG           |
| 312                  | GCCSINGAWD         | CGC           | GCA           |
| 452                  | TYESASSRFQEG       | CGC           | GCG, GAA, or CAA |
| 453                  | TYESASSRFLHG       | CGC           | GCG, GAA, or CAA |
| 458                  | FOEGRVDRSAPTEL    | CGG           | GCG           |
| 463                  | FLHGRVDBAATEL     | AGG           | GCG           |
| 498                  | FGRVDBAATEL       | AGG           | GCG           |

* R, rat; D, Drosophila; N, nematode.
* GAA, glutamate; CAA, glutamine.
* This mutation used only in the R452/R453 double mutant.

Conserved arginines in rat choline acetyltransferase and their conversion to alanine.

Table I

- R, rat; D, Drosophila; N, nematode.
- GAA, glutamate; CAA, glutamine.
- This mutation used only in the R452/R453 double mutant.

The kinetic properties of the mutant enzymes were initially analyzed in E. coli extracts rather than with purified enzyme. The kinetic properties of the mutant enzymes were initially compared with the wild type enzyme in the presence of 0.25 mM NaCl, conditions previously shown to give maximal activity (25). The results from this kinetic analysis are summarized in Table I. Comparing the various alanine substitutions, the most significant effect observed is with the mutant R452A in which the $K_m$ for CoA increases more than 12-fold, the $k_{cat}$ increases 8-fold, and $k_{cat}/K_m$ increases 7-fold.
Fig. 1. SDS-PAGE analysis of purified recombinant ChAT mutants. 5 μg of the indicated enzyme was analyzed on an SDS-PAGE gel as described under “Experimental Procedures.”

| Mutant      | KM(COA) | KM(ACh) | KM(GAC) | kcat    |
|-------------|---------|---------|---------|---------|
| Wild type   | 3.8 ± 0.1| 0.9 ± 0.1| 39 ± 3  | 1147 ± 32|
| R99A        | 3.9 ± 0.5| 0.7 ± 0.1| 49 ± 3  | 1796 ± 61 |
| R250A       | 2.6 ± 0.4| 1.5 ± 0.3| 36 ± 3  | 1706 ± 82 |
| R312A       | 4.3 ± 0.6| 1.3 ± 0.1| 51 ± 5  | 1312 ± 55 |
| R452A       | 42 ± 4.3 | 7.6 ± 1.1| 67 ± 4  | 7630 ± 557|
| R452Q       | 44 ± 1.2 | 10 ± 3   | 89 ± 12 | 7090 ± 640|
| R452E       | 185 ± 2.6| 54 ± 5   | 100 ± 4 | 15,060 ± 620|
| R453A       | 8.3 ± 0.2| 4.4 ± 0.5| 72 ± 5  | 2624 ± 92 |
| R453E       | 26 ± 1.7 | 11.2 ± 1.2| 51 ± 5 | 5460 ± 220 |
| R458A       | 6.2 ± 0.2| 2.0 ± 0.3| 162 ± 18| 3110 ± 165|
| R463A       | 9.3 ± 0.6| 2.4 ± 0.4| 114 ± 14| 1604 ± 112|
| R452Q/R453Q | 581 ± 56 | 69 ± 1.9 | 141 ± 23| 18,355 ± 760|

* ACh, acetylcholine.
* dCoA, dephospho-CoA.
* Enzyme protein estimated by Western blot analysis.

**TABLE III**

| Mutant      | KM(COA) | KM(ACh) | KM(GAC) | kcat    |
|-------------|---------|---------|---------|---------|
| Wild type   | 0.25 ± 0.03| 0.4 ± 0.06| 27 ± 2  | 595 ± 45 |
| R452A       | 15.4 ± 2  | 9.2 ± 0.7| 50 ± 4  | 8621 ± 426|
| R453A       | 1.0 ± 0.1 | 1.5 ± 0.1| 76 ± 5  | 3674 ± 290|
| R458A       | 0.4 ± 0.08 | 2.0 ± 0.03 | 144 ± 11 | 4541 ± 328 |
| R463A       | 1.6 ± 0.3 | 1.1 ± 0.05 | 83 ± 6  | 1267 ± 100 |

*a ACh, acetylcholine.
*b dCoA, dephospho-CoA.

to be a likely candidate as an active site residue, with arginines 453, 458, and 463 also being possible candidates. It has been previously observed that anions affect the kinetic parameters of the ChAT reaction (26). That is, anions act to increase Vmax but at the same time increase the K uninsured for both substrates (25). It has been suggested that this effect can be attributed, at least in part, to anions interfering with the binding of the 3'-phosphate of coenzyme A to an active site arginine (9). Thus a similar kinetic analysis was conducted with the wild type enzyme and the four candidate active site arginine mutants under conditions of low ionic strength. The results of this analysis are shown in Table III. In agreement with previous studies (25, 26), it can be seen that with the wild type enzyme decreasing the anion concentration decreases the K uninsured for CoA –12-fold but decreases the K uninsured for choline less than 3-fold and lowers kcat by approximately 2-fold. In contrast decreasing salt has little effect on the K uninsured for dephospho-CoA, a finding consistent with the proposal that anions compete for the interaction at the 3'-phosphate of coenzyme A with an arginine.

The effects of decreased ionic strength are different with the R452A mutant. The K uninsured for CoA is reduced only –3-fold by lowering the ionic strength of the assay, while there is little change in the K uninsured for acetylcholine and kcat is essentially unchanged. As with the wild type enzyme the K uninsured for dephospho-CoA is barely affected by changes in anion concentration. The other putative active site arginine mutants all exhibit changes in their kinetic properties that are similar to those observed with the wild type enzyme except that kcat remained unchanged. Again these results are consistent with arginine 452 as the active site arginine interacting with CoA.

In order to test for a change in kinetic mechanism between the wild type enzyme and the R452A mutant we determined the inhibition pattern with choline as a product inhibitor. The ChAT reaction follows primarily a Theorell-Chance kinetic mechanism, characterized by competitive inhibition between the inner substrate pair choline and acetylcholine (25). As shown in Fig. 2, choline acts as a competitive inhibitor with respect to acetylcholine for both the wild type enzyme and for the R452A mutant, a finding suggesting that both enzymes exhibit the same Theorell-Chance kinetic mechanism. However, the K uninsured for choline was greater for the mutant (1.1 mM) as compared with the wild type enzyme (0.1 mM). Although not shown, competitive inhibition between choline and acetylcholine was also observed with the most severely affected mutant R452Q/R453Q with the Ki for choline increased to 12 mM.

We next analyzed the ability of each of the alanine mutants to react with the arginine-specific reagent phenylglyoxal. As shown in Fig. 3 treatment of the wild type enzyme with 5 mM phenylglyoxal results in biphasic inactivation. There is a rapid initial decline of activity, which is too fast to measure but which results in a loss of ~35% enzyme activity. This is followed by a slower loss in enzyme activity, which plateaus at ~90% inactivation. A replottting the data as log (% activity remaining)
versus time from 0.1 to 45 min is linear assuming an end point of 90% inactivation. From this analysis a half-time of ~11 min was obtained. As shown in Fig. 3 the slow phase of inactivation was prevented by inclusion of acetyl-CoA in the inactivation reaction at a concentration in the range of the $K_m$ for CoA. Acetylcholine was without effect. Inclusion of acetyl-CoA at concentrations greater than 50 times the $K_m$ for CoA had no effect on the fast phase (data not shown). Conversion of arginines 99, 312, and 453 to alanine had no significant effect on phenylglyoxal inactivation. In each of these cases the same extent of enzyme inactivation was observed in the rapid phase, and the half-time for the slow phase varied only slightly from that observed with the wild type enzyme (10, 12, and 10 min for alanine substitutions at arginines 99, 312, and 453, respectively).

Changing arginine 452 to alanine resulted in a biphasic inactivation curve similar to the wild type enzyme except that the secondary phase was faster ($t_{0.5} \sim 7$ min) and inactivation...
went to completion. Fig. 3. Acetyl-CoA at the same concentration used to protect the wild type enzyme from inactivation was ineffective with this mutant; however, increasing the acetyl-CoA concentration to 13.6 μM was able to afford protection (Fig. 3). Although not shown, acetylcholine at 15 mM had no effect on phenylglyoxyal inactivation.

Changing arginine 463 to Ala resulted in a considerably more rapid rate of inactivation by phenylglyoxyal (Fig. 3). In this case the initial rapid and secondary phases of the reaction could not be distinguished. However, using 2.5 μM acetyl-CoA to protect against inactivation, the biphasic nature of the inactivation process became apparent (Fig. 3). Changing Arg-250 to Ala totally eliminated the secondary phase of inactivation by phenylglyoxyal (Fig. 3).

In order to provide additional evidence that Arg-452 is involved in CoA binding, we measured the ability of coenzyme A to protect the enzyme against thermal inactivation. The wild type enzyme is inactivated at 48 °C under low ionic strength conditions with a $t_{0.5}$ of 60 s. As shown in Fig. 4, CoA afforded partial protection against thermal inactivation exhibiting a $K_d$ value of –0.4 μM, a value similar to the kinetic $K_m$ of 0.25 μM listed in Table II. The R452A mutant was more temperature stable inactivated at 48 °C with a $t_{0.5}$ of –20 s. At 44 °C the $t_{0.5}$ was 35 s, and CoA also provided partial protection against thermal inactivation; however, in this case the estimated binding constant was shifted to –6 μM.

**DISCUSSION**

Previous studies have shown that the enzyme choline acetyltransferase is inactivated by arginine-specific reagents in a reaction protected by the substrate acetyl-CoA (9). This observation, in conjunction with the results of kinetic studies which showed that dephospho-coenzyme A was poorly bound by the enzyme (6), led to the proposal of an active site arginine that interacts with the 3'-phosphate of coenzyme A. Since the results of chemical modification experiments are often equivocal or ambiguous, we utilized alanine scanning to systematically search for this active site arginine by replacing seven conserved arginines with alanine. The results of the kinetic analysis of these mutant enzymes are consistent with arginine 452 being the likely candidate. The predicted properties of such a mutant would include a decreased affinity for CoA with little change in the affinity of the mutant for dephospho-CoA. In accordance with this predicted behavior conversion of arginine 452 to alanine, glutamine, or even glutamate caused a 12–50-fold increase in the $K_m$ for CoA but less than a 3-fold change in the $K_m$ for dephospho-CoA. Thus in contrast to the wild type enzyme, where the $K_m$ for CoA is –10-fold lower than the $K_m$ for dephospho-CoA, the $K_m$ values for CoA and dephospho-CoA are nearly the same in R452A and R452Q. With R452E dephospho-CoA becomes a better substrate than CoA. The observation that the properties of R452A and R452Q are quite similar rules out any complications that might have been introduced as a result of a change in the side chain volume when substituting the non-isosteric alanine for arginine. Thus this data is consistent with arginine 452 interacting with the 3'-phosphate of coenzyme A.

The effects of changing arginine 452 by mutagenesis may be blunted by the presence of an adjacent arginine in position 453. Arginine 453 may also be directly involved in coenzyme binding or alternatively may realign in the Arg-452 mutants so that it can participate in coenzyme binding. This might explain why the most dramatic effects are seen with the R452Q/R453Q double mutant in which both arginines have been substituted.

At first glance it might seem surprising that the $K_m$ for both CoA and choline as well as $k_{cat}$ would be increased by mutations at position 452. In the Theorell-Chance kinetic mechanism, which the Arg-452 mutant appears to obey, the rate-limiting step in the reaction is the release of product from the enzyme, $k_s$ in Scheme 1. The $K_m$ values for coenzyme A and acetylcholine reflect their respective affinity in the steady state and are defined as the ratio of $k_{-1}/k_1$ and $k_{-3}/k_3$, respectively. The data are consistent with mutations at Arg-452 decreasing the affinity of the enzyme for CoA (or acetyl-CoA) by loss of the interaction with the 3'-phosphate. Kinetically this is manifested as an increase in the rate constant $k_s$ ($k_{cat}$), which represents dissociation of the coenzyme from the enzyme. An increase in $k_s$ would also be expected to result in an increase in $K_{m(acetylcholine)}$ since this kinetic constant is directly proportional to $k_s$. With R452A and R452Q there is a constant 7–12-fold increase in $k_{cat}$, $K_{m(acetylcholine)}$, and $K_{m(CoA)}$, a finding consistent with this proposal. The greater effects on $K_{m(acetylcholine)}$ and $K_{m(CoA)}$ than on $k_{cat}$ as seen in R452E and R452Q/R453Q may reflect other structural changes in the enzyme or may be due to the presence of inactive enzyme since...
the activity of these mutants was found to be rather labile. The increase in kinetic constants seen in R453E could likely result from ionic interactions between the introduced glutamate and the arginine at position 452. This would result in a decreased interaction of arginine 452 with the coenzyme, making this mutant resemble an Arg-452 mutant.

In low salt the $K_m$ for dephospho-CoA decreases $12$-fold, while the $K_m$ for CoA and eliminates the increase in $V_{max}$. If as postulated, $K_m$ is increased by anions as a result of disrupting the interaction of an active site arginine with the 3'-phosphate of CoA, this effect would be expected to be eliminated when arginine is converted to alanine. Thus, as predicted, the R452A mutant, in contrast to the wild type enzyme, is refractory to the extent of inactivation by phenylglyoxal. This indicates that the residue which reacts with phenylglyoxal appears to be arginine 250. Changing this residue to alanine has no effect on the kinetic properties of the enzyme, indicating that its reaction with phenylglyoxal results in either a conformational change or steric hindrance. Modification of this residue does not result in a loss in activity, supporting the conclusion that this is not a critical residue for catalysis. Changing arginine 452 or 463 to alanine increased the rate as well as the extent of inactivation by phenylglyoxal. This indicates that these mutations permitted either an additional residue to react with phenylglyoxal or resulted in a structural change such that reaction with arginine 250 totally blocked activity.

Interestingly, conversion of three other arginines, Arg-453, -458, and -463, to alanine also affects the kinetics of the reaction, albeit to a considerably lesser extent. These arginines are clustered in the primary sequence. Thus it is possible that these residues are situated in the vicinity of or actually constitute part of the active site in three-dimensional space, participating in coenzyme A binding. It is worth noting that x-ray crystallographic studies have shown that there are multiple interactions such as electrostatic interactions, hydrophobic interactions, and hydrogen bonds between coenzyme A and the enzyme dimerate synthase (27). At least three arginines form salt bridges with the three charged phosphate moieties; Arg-46 and Arg-324 (through a water molecule) interact with the 5'-diphosphate while Arg-164 interacts with the 3'-phosphate in addition to possible hydrogen bonding interactions.

In summary alanine scanning of conserved arginines in the choline acetyltransferase reaction has provided evidence for arginine 452 being an active site residue that functions by interacting with the 3'-phosphate of the substrate CoA/acetyl-CoA. This arginine lies within a highly conserved region of the enzyme, whose sequence varies little among such divergent species as mammals, Drosophila, and the nematode, C. elegans. Direct evidence for this proposal must await the determination of the three-dimensional structure of the enzyme.

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