Substituted Cysteine Accessibility of the Third Transmembrane Domain of the Creatine Transporter

DEFINING A TRANSPORT PATHWAY

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Twenty-two amino acid residues from transmembrane domain 3 of the creatine transporter were replaced, one at a time, with cysteine. The background for mutagenesis was a C144S mutant retaining ~75% of wild-type transport activity but resistant to methanethiosulfonate (MTS) reagents. Each substitution mutant was tested for creatine transport activity and sensitivity to the following MTS reagents: 2-aminoethyl methanethiosulfonate (MTSEA), 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET), and 2-sulfonatoethyl methanethiosulfonate (MTSES). Two mutants (G134C and Y148C) were inactive, but most mutants showed significant levels of creatine transport. Treatment with MTSEA inhibited the activity of the W154C, Y147C, and I140C mutants. Creatine partially protected I140C from inactivation, and this residue, like Cys-144 in the wild-type CreaT, is predicted to be close to a creatine binding site. MTSEA inactivation of Y147C was dependent on Na+ and Cl− suggesting that solvent accessibility was ion-dependent. Helical wheel and helical net projections indicate that the three MTSEA-sensitive mutants (W154C, Y147C, and I140C) and two inactive mutants (V151C and Y148C) are aligned on a face of an α-helix, suggesting that they form part of a substrate pathway. The W154C mutant, located near the external face of the membrane, was accessible to the larger MTS reagents, whereas those implicated in creatine binding were only accessible to the smaller MTSEA. Consideration of our data, together with a study on the serotonin transporter (Chen, J. G., Sachpatzidis, A., and Rudnick, G. (1997) J. Biol. Chem. 272, 28321–28327), suggests that involvement of residues from transmembrane domain 3 is a common feature of the substrate pathway of Na+- and Cl−-dependent neurotransmitter transporters.

Creatine is present at high concentrations in tissues with large and fluctuating demands for energy such as heart, skeletal muscle, and brain. Creatine is converted to phosphocreatine by creatine kinase following ATP synthesis in mitochondria. Phosphocreatine is used to recycle ATP by reversal of this reaction at sites of high energy utilization. In mammals, creatine is either obtained by synthesis from sequential reactions occurring in the kidney and liver or from the diet. Interestingly, tissues that contain high levels of creatine/creatine do not appear to synthesize their own creatine (1). A specific uptake system for creatine transport activity and sensitivity to the following MTS reagents: 2-aminoethyl methanethiosulfonate (MTSEA), 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET), and 2-sulfonatoethyl methanethiosulfonate (MTSES). Two mutants (G134C and Y148C) were inactive, but most mutants showed significant levels of creatine transport. Treatment with MTSEA inhibited the activity of the W154C, Y147C, and I140C mutants. Creatine partially protected I140C from inactivation, and this residue, like Cys-144 in the wild-type CreaT, is predicted to be close to a creatine binding site. MTSEA inactivation of Y147C was dependent on Na+ and Cl− suggesting that solvent accessibility was ion-dependent. Helical wheel and helical net projections indicate that the three MTSEA-sensitive mutants (W154C, Y147C, and I140C) and two inactive mutants (V151C and Y148C) are aligned on a face of an α-helix, suggesting that they form part of a substrate pathway. The W154C mutant, located near the external face of the membrane, was accessible to the larger MTS reagents, whereas those implicated in creatine binding were only accessible to the smaller MTSEA. Consideration of our data, together with a study on the serotonin transporter (Chen, J. G., Sachpatzidis, A., and Rudnick, G. (1997) J. Biol. Chem. 272, 28321–28327), suggests that involvement of residues from transmembrane domain 3 is a common feature of the substrate pathway of Na+- and Cl−-dependent neurotransmitter transporters.

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2 The abbreviations used are: CreaT, creatine transporter; GABA, γ-aminobutyric acid; GAT, γ-aminobutyric acid transporter 1; KRH, Krebs-Ringer-HEPES buffer; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSET, 2-sulfonatoethyl methanethiosulfonate; MTSES, 2-(trimethylammonium)ethyl methanethiosulfonate; SERT, serotonin transporter; SLC6, solute carrier family 6; TM, transmembrane domain; WT, wild-type.
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tivated by MTSET. Cocaine and serotonin protected the I172C SERT mutant from MTSET inactivation suggesting that Ile-172 is located in a permeation pathway close to the substrate binding site (21).

We found previously that exposure of Cys-144 in TM3 to low concentrations of MTSEA led to rapid inactivation of the CreaT (22). Creatine was able to protect the CreaT from MTSEA inactivation suggesting that this residue is close to a substrate binding site. A C144S mutant retained ~75% of the creatine transporter activity but was resistant to inactivation. To help define the transport pathway for creatine we have used this mutant as a background for the substitution of 22 residues in TM3 one at a time for cysteine and to test for sensitivity to MTS reagents.

EXPERIMENTAL PROCEDURES

Mutagenesis—Mutant transporters were generated by site-directed mutagenesis of the C144S CreaT mutant (22) using the QuickChange\textsuperscript{TM} kit (Stratagene, La Jolla, CA). The mutated cDNA was excised from pBluescript K5 and subcloned into pcDNA3.1+ (Invitrogen). The cDNA sequence of all CreaT mutants was confirmed by DNA sequencing. The construction of wild-type bovine CreaT cDNA in pcDNA3.1+ has been described previously (22).

Expression of Mutant CreaTs—HEK293 cells were grown in minimum Eagle’s medium containing 10% fetal bovine serum, streptomycin, and penicillin and seeded at a density of 1.2 × 10\(^5\) cells/well in 12-well (3.8-cm\(^2\) area) poly-L-lysine (Sigma, P1399)-coated plastic culture dishes (Falcon) 48 h prior to transfection. Cells were transfected with 0.4 

μg of plasmid and 4 μl of Lipofectamine 2000\textsuperscript{TM} per well following procedures recommended by the manufacturer (Invitrogen). For biotinylation experiments, cells were plated out at a density of 3 × 10\(^5\) cells/well in 6-well (9.6-cm\(^2\) area) poly-L-lysine-coated plastic culture dishes, and the transfection procedure was scaled up accordingly.

Creatine Transport Assay—Cells expressing CreaT mutants were assayed for creatine uptake activity 24 h after transfection. Medium was removed from wells, and the cells were washed with 1 ml of prewarmed KRH (Krebs-Ringer-HEPES) uptake buffer. The KRH buffer contained 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 25 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with NaOH. The cells were incubated for 3 min at 37 °C with 1 ml of KRH containing 20 μl of [4-\(^{14}\)C]creatine (American Radiolabeled Chemicals, ARC-176, 55 μCi/mmole, diluted to a specific activity of 1 mCi/mmole). After the incubation the uptake medium was removed by aspiration, and the cells were washed three times with 1 ml of ice-cold KRH buffer. Cells were solubilized in 0.5% Triton X-100, and aliquots were taken for scintillation counting. Samples were also taken to determine protein concentration using a detergent-compatible protein assay (Bio-Rad) with bovine serum albumin as standard. All assays were carried out in triplicate.

Treatment with MTSEA, MTSET, and MTSES—MTS reagents were obtained from Toronto Research Chemicals, Ontario, Canada. Cells were incubated with 0.25 mM MTSEA, 1 mM MTSET, or 10 mM MTSES in KRH buffer for 5 min prior to creatine transport assays. Cells were washed once with 1 ml of KRH prior to assay. To study the kinetics of inactivation, cells expressing some mutants were incubated with MTSEA at the concentrations and times indicated under “Results” and in the legend for Fig. 4. In some cases cells were incubated with MTS reagents in the presence of creatine as indicated in the text. KRH buffer was prepared without Na\(^+\) (using N-methylglucamine) or without Cl\(^-\) (using sodium gluconate) to investigate the effect of these ions on MTSEA inactivation or creatine protection.

Cell Surface Biotinylation—Cell surface expression of the transporters was determined using a membrane-impermeable biotinylation reagent, sulfo-NHS-SS-biotin (Pierce), as described previously (22). Biotinylated proteins were purified on NeutrAvidin\textsuperscript{TM} beads (Pierce) and extracted from the beads by adding 50 μl of 2 × SDS reducing buffer (125 mM Tris, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) and incubating for 1 h at 37 °C followed by centrifugation at 10,000 × g for 5 min. The biotinylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with affinity-purified C-terminal-directed antibodies against the CreaT (22).

RESULTS

Transport Activity of Cysteine Mutants—Replacement of Cys-144 in TM3 (Fig. 1) of the bovine CreaT with a serine gives a mutant (C144S) that retains ~75% of the activity of the wild-type CreaT but is resistant to treatment with 0.25 mM MTSEA, 1 mM MTSET, or 10 mM MTSES (22). This mutant was used as a background to construct 22 cysteine replacement mutants. The transport activity of these mutants varied widely (Fig. 2A). Two mutants (G134C and Y148C) were inactive, and the activity of V151C was too low to study further. Three other mutants (A136C, Y147C, and W154C) had severely reduced activity (~25% of C144S). Three mutants retained 25–50% activity, and 13 mutants had levels of activity ≥50% of C144S.

Surface Expression of TM3 Mutants—To investigate whether variations in transport activity resulted from differences in cell surface expression, cells expressing each TM3 mutant were labeled with sulfo-NHS-SS-biotin. Biotinylated proteins were purified from cell lysates with NeutrAvidin\textsuperscript{TM} beads and analyzed by Western blotting with an antibody to the C-terminal region of the transporter. All of the cysteine mutants and the background C144S mutant showed the presence of a diffuse ~120-kDa band corresponding to biotinylated CreaT (Fig. 2B, lower panel). This band was absent in cells transfected with empty vector (Fig. 2B, lower panel, pc). The expression of the biotinylated ~120-kDa band for each mutant was normalized relative to WT CreaT (Fig. 2B, upper panel). There was some variability in surface expression relative to the C144S background, although this did not correlate with levels of activity. It appears likely that differences in transport activity result from cysteine substitution rather than altered expression, folding, or trafficking of the transporter to the plasma membrane.

Sensitivity of TM3 Mutants to MTS Reagents—To determine whether residues in TM3 were accessible to MTS reagents, all of the TM3 cysteine mutants in the C144S CreaT background were reacted with MTSEA and the larger MTSET (positively charged) and MTSES (negatively charged) reagents (Fig. 3). Three mutants (W154C, Y147C, and I140C) were inhibited to a significant extent (>40%) by a 5-min treat-

FIGURE 1. Schematic diagram of TM3 of the CreaT. The diagram shows the sequence (Leu-133 to Trp-154) corresponding to the predicted TM region. The cysteine replaced by serine to generate the C144S mutant used for mutagenesis is highlighted by gray shading. The orientation of the α-helix relative to the outside and inside of the cell is also shown. C-term, C terminus; N-term, N terminus.
ment with 0.25 mM MTSEA, a concentration five times higher than required to inhibit WT CreaT (22). W154C was the only mutant inhibited by a 5-min treatment with 1 mM MTSET (76%) and 10 mM MTSES (70%), respectively. Reactivity with MTSET and MTSES appeared to be limited to residues predicted to be near the exterior end of TM3 (Fig. 1).

To determine the relative reactivity of the W154C, Y147C, and I140C mutants, we determined the concentration dependence and time course of MTSEA inactivation. These data are compared with results from the WT CreaT and the background (C144S) and A136C mutants (Fig. 4). Increasing concentrations of MTSEA resulted in an exponential decrease in the activity of I140C, W154C, and WT CreaT. However, maximum inactivation of I140C (∼90%) and W154C (∼80%) required an ∼10-fold higher concentration of MTSEA than the WT CreaT required. The $t_{1/2}$ for inactivation of WT CreaT was 0.47 ± 0.03 min with 50 μM MTSEA, and the $t_{1/2}$ values for the I140C and W154C mutants were 0.81 ± 0.06 and 0.82 ± 0.12 min, respectively, with 0.5 mM MTSEA (Fig. 4B). The C144S background and the A136C mutants showed much smaller and apparently linear decreases in activity with increasing concentrations of MTSEA. We attributed the decline in creatine transport seen in these mutants to result from either slow reactivity of other cysteine residues in the CreaT or toxicity from the higher concentrations of MTSEA. We observed that MTSEA was toxic to HEK293 cells, resulting in the detachment of the cells from the culture dishes. The Y147C mutant exhibited a low level of transport activity that was partially sensitive (40–50% inactivation) to MTSEA (Fig. 4). We estimated a $t_{1/2}$ for inactivation of 3.7 ± 0.12 min for this mutant with 0.5 mM MTSEA. However, the low activity and slow reactivity of this mutant, combined with difficulties in using higher concentrations of MTSEA, limited further analysis of this mutant. The data shown in Fig. 4B were used to determine the pseudo second order rate constants for the inactivation of WT CreaT (Cys-144), I140C, W154C, and Y147C as...
29,760 ± 1302, 1688 ± 107, 1754 ± 205, and 388 ± 170 (min⁻¹ M⁻¹, mean ± S.E.), respectively.

Effect of Na⁺, Cl⁻, and Creatine on the Sensitivity of Cysteine Replacement Mutants to MTSEA—Experiments with the WT CreaT confirmed previous work (22). MTSEA inactivation was independent of Na⁺ and Cl⁻, whereas the ~90% protection from inactivation by 1 mM creatine was reduced in the absence of these ions (Fig. 5). The background C144S was unaffected by MTSEA or the presence or absence of Na⁺, Cl⁻, or creatine. Inactivation of the I140C mutant (~64%) by MTSEA was also not dependent on Na⁺ or Cl⁻. However, the partial protection from creatine (1 mM) was reduced by the absence of either ion. The concentration dependence for the protection by creatine from MTSEA inactivation was determined for both WT CreaT and I140C (Fig. 6). Approximately twice the concentration of creatine was required to protect I140C by 50% compared with the WT CreaT (Kᵣ values for creatine binding of 0.69 and 0.31 mM creatine, respectively). The Kᵣ values for creatine transport of I140C and WT CreaT were 0.212 and 0.079 mM, respectively (data not shown).

Inactivation of W154C with MTSEA was independent of Na⁺ and Cl⁻ and not protected by the presence of creatine, consistent with this residue being located away from the substrate binding site. In contrast to WT CreaT or other cysteine mutants, MTSEA inactivation of Y147C was dependent on Na⁺ and Cl⁻. Protection from inactivation by creatine (~46%) was also reduced in the absence of either Na⁺ or Cl⁻. This suggests that solvent accessibility of Y147C is also ion-dependent.

DISCUSSION

We have tested the functional importance and aqueous accessibility of amino acid residues in TM3 of the CreaT by the substituted cysteine accessibility method. An ideal starting point for this study would have been the engineering of a Cys-less protein with normal function (20). However, the bovine CreaT contains 22 Cys residues, so it is unlikely that an active Cys-less mutant could be generated. We used a C144S mutant that retains 75% of wild-type activity and was known to be relatively resistant to MTS reagents as a background for the mutagenesis study (22). Although this proved valuable, there were some limitations to the use of this mutant. Exposure to increasing concentrations of MTSEA led to decreases in the activity of the background mutant. This may have occurred from reactivity of some of the remaining 21 Cys residues of the CreaT or toxicity because of modification of other proteins in the HEK293 cells. Experimentally, we were able to compensate for these difficulties by use of appropriate controls, relatively short (5
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The complete scanning mutagenesis of TM3 of the CreaT provides an important opportunity for comparison with a similar study of the SERT (21). Cysteine substitution mutants that are inactive in both transporters would indicate positions critical for a common function. The sequence of TM3 of the CreaT and the equivalent region of the SERT are compared in Fig. 7A. Conversion of Tyr-148 of the CreaT and Tyr-176 of the SERT to cysteine renders both transporters inactive. This agrees with the previous suggestion that this residue may be part of a substrate binding site (21) and the finding that even conservative mutations at this position (Tyr-140) of GAT-1 resulted in the loss of activity (19). V151C of the CreaT had no detectable activity, whereas the equivalent SERT mutant I179C retained 50–70% of the control activity. Although this indicates a difference between the two transporters, the SERT was found to be less tolerant to other mutations, such as replacement of this Ile with Ser or Ala (21). Modification of I179C by MTSET was shown to inhibit transport activity but not substrate binding. It was suggested that Ile-179 is located in a conformationally sensitive region, closer to the external medium where its accessibility is increased by substrate binding (23). Ile-155 in the norepinephrine transporter (equivalent to SERT Ile-179) was also present in a conformationally sensitive region of TM3 (23). We found G134C CreaT to be inactive. This residue is conserved in the SERT, but studies of mutants of this position have not been reported.

There is good agreement that the position equivalent to Cys-144 of the CreaT and Ile-172 of the SERT is close to a substrate binding site (21, 22). MTSET inactivation of I172C SERT was blocked by both serotonin and cocaine, and creatine protected the CreaT from MTSEA inactivation. Close proximity to the binding site is consistent with the size of the side chain at this position affecting substrate specificity in the CreaT. A C144L CreaT mutant had a higher $K_d$ for creatine and, unlike WT CreaT, was inhibited competitively by GABA (22). The identification of Cys-144 as the residue modified by MTSEA is supported by the resistance of the CreaT mutants C144S and C144A (22) and C144L to MTSEA.

Activity screening of CreaT cysteine replacement mutants following a 5-min treatment with 0.25 mM MTSEA showed substantial inactivation (≥40%) of the W154C, Y147C, and I140C mutants. The kinetics of MTSEA inactivation (concentration and time dependence) at these mutants was determined and used to determine their relative accessibility compared with the WT CreaT, where MTSEA reactivity is presumed to occur at Cys-144. Surprisingly, the reactivity as determined from the pseudo second order rate constants was ~17 times greater for W154C and W154C were similar to that determined for inactivation of the

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SERT (782 min$^{-1}$ m$^{-1}$) by MTSET (21). Thus, the apparent reactivity of Cys-144 appears to be greater than other solvent-accessible regions of TM3 of the SERT and the CreaT. The most likely explanation would be if the microenvironment of Cys-144 results in a lower pK$\alpha$ value for this cysteine. The ionized thiolate group is known to react much faster than SH-groups with MTS reagents (20).

Experiments on the protection by creatine from MTSEA inactivation revealed some differences between the CreaT and the SERT. Protection of I172C of the SERT from MTSET inactivation by serotonin was not dependent on temperature or Na$^+$ suggesting that it resulted from direct occlusion and was not secondary to a substrate-induced conformational change (21). However, results obtained previously (22) and in Fig. 5 show that protection by creatine from MTSEA inactivation of both the Cys-144 of WT CreaT and the I140C mutant is reduced in the absence of Na$^+$ and Cl$^-$. Although these ions do not appear to affect the solvent accessibility of these positions, it appears that Na$^+$ and/or Cl$^-$ may be required for creatine binding. We found that higher concentrations of creatine were required to prevent reactivity of I140C with MTSE reagents. MTSEA (66 Å$^3$) is of a similar size to the CreaT substrate creatine and 3-guanidino propionate, whereas MTSET, which was used for studies of the SERT, is significantly larger (10 Å$^3$).

We have shown that residues within TM3 of the creatine transporter form part of a substrate transport pathway. This is likely to extend from Trp-154 to Ile-140. The binding of creatine may depend on Na$^+$ - and Cl$^-$-dependent conformational changes. Other regions of the transporter must also contribute to the binding site. Recently the aqueous accessibility of TM1 of the SERT and GAT-1 has been shown to be modulated by substrates (24, 25). Also TM1s 1 and 2 of GAT-4 have been shown to contain molecular determinants of substrate specificity (26). Further studies are required to see how these and other TM3s are oriented in relation to TM3.

The CreaT is a transporter required for the uptake of a substrate important for energy metabolism, a function seemingly distinct from neurotransmitter transport. However, our data together with studies of the SERT (21), suggest involvement of residues from TM3 to be a common feature of substrate pathways for other members of the Na$^+$ - and Cl$^-$-dependent neurotransmitter transporter (SLC6) family.

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