Cysteine 144 in the Third Transmembrane Domain of the Creatine Transporter Is Located Close to a Substrate-binding Site*

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All creatine transporters contain a cysteine residue (Cys$^{144}$) in the third transmembrane domain that is not present in other members of the Na$^+$/Ca$^2+$-dependent family of neurotransmitter transporters. Site-directed mutagenesis and reaction with methane thiosulfonates were used to investigate the importance of Cys$^{144}$ for transporter function. Replacement of Cys$^{144}$ with Ser did not significantly affect the kinetics or activity of the transporter, whereas a C144A mutant had a higher $K_m$ (0.33 compared with 0.18 mM). Substitution of Cys$^{144}$ with Leu gave a mutant with a 5-fold higher $K_m$ and a reduced specificity for substrate. Low concentrations of 2-aminoethyl methanethiosulfonate (MTSEA) resulted in rapid inactivation of the creatine transporter. The C144S mutant was resistant to inactivation, indicating that modification of Cys$^{144}$ was responsible for the loss of transport activity. Creatine and analogues that function as substrates of the creatine transporter were able to protect from MTSEA inactivation. Na$^+$ and Cl$^-$ ions were not necessary for MTSEA inactivation, but Na$^+$ was found to be important for creatine protection from inactivation. Our results indicate that cysteine 144 is close to the binding site or part of a permeation channel for creatine.

Creatine and creatine phosphate are essential for the maintenance of ATP levels in tissues with high and fluctuating energy demands such as skeletal muscle and brain (1–3). Creatine kinase catalyzes the reversible transfer of a phosphate from creatine phosphate to ADP regenerating ATP. In mammals, creatine is either synthesized by sequential reactions occurring in the kidney and liver or obtained from the diet. A specific uptake system for creatine has been demonstrated in skeletal muscle (4), some cultured cell preparations (5), human monocytes and macrophages (6), and astroglial-rich cultures from neonatal rats and mice (7).

Molecular cloning studies have identified muscle and brain cDNAs encoding high affinity sodium- and chloride-dependent creatine transporters (8). The deduced sequence of the rabbit CreaT$^+$ exhibits significant homology to the Na$^+$ and Cl$^-$-dependent GABA/norepinephrine (GAT-1/norepinephrine transporter) gene family of neurotransmitter transporters (9, 10). All members of this family are predicted to contain 12 membrane-spanning domains, to contain a large extracellular loop containing sites for N-linked glycosylation between the third and fourth transmembrane domains, and to have the amino and carboxyl termini facing the cytoplasmic side of the membrane.

In the absence of a three-dimensional structure, indirect approaches have been used to gain insight into the structure and function of transporter proteins. The identification of residues required for substrate selectivity and specificity is particularly important. Chimeras were prepared by exchanging domains between the norepinephrine and dopamine transporters, two transporters that have high sequence similarity but differ in their inhibitor sensitivity. One group suggested that regions spanning TM1–3, 11, and 12 were required for substrate affinity (11), whereas another found that TMs 8–12 were dominant for substrate selectivity and specificity (12). Site-directed mutagenesis has also been useful in identifying amino acid residues involved in substrate binding. Tyr$^{146}$ in TM3 of the GABA transporter (GAT-1) was found to be critical for substrate recognition and transport (13). Replacement of the equivalent residue in the SerT (Tyr$^{176}$) with cysteine resulted in a reduced affinity for serotonin and cocaine (14). The tyrosine at a position equivalent to 140 of GAT-1 is conserved in all transporters, including the CreaT, and it has been suggested that this residue may be required for a common function (13).

Methanethiosulfonate (MTS) reagents are useful tools to identify residues that may be important for substrate binding and the specificity of neurotransmitter transporters. If cysteine residues are exposed to the external medium or part of a water-accessible channel or binding site, then they will react with polar MTS reagents (15). Two MTS derivatives, 2-aminoethyl methanethiosulfonate (MTSEA) and 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET), are positively charged, whereas 2-sulfonatoethyl methanethiosulfonate (MTSES) is negatively charged. Mutants of the SerT in which the Ile at positions 172 and 179 in TM3 was replaced with cysteine were readily inactivated by MTSET (14). The I172C mutant was protected from MTSET inactivation by substrate, suggesting that it was located in a permeation pathway close to the substrate-binding site (16). This protection was not dependent on temperature or Na$^+$, suggesting that it results from direct occlusion of I172C by serotonin and not secondary to a substrate-induced conformational change. In contrast, Ile$^{179}$ of the SerT and the equivalent residue of the norepinephrine transporter, Ile$^{155}$, were found to be in a conformationally sensitive region of TM3.

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The abbreviations used are: CreaT, creatine transporter; GAT-1, γ-aminobutyric acid transporter 1; GABA, γ-aminobutyric acid, KRH, Krebs-Ringer-Hepes buffer; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSES, 2-sulfonatoethyl methane-

thiosulfonate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate; PBS, phosphate-buffered saline; SerT, serotonin transporter; TM, transmembrane domain; wt, wild-type; GST, glutathione S-transferase.
A comparison of the protein sequence of transporters separated by a large phylogenetic distance may also help identify candidate amino acid residues required for substrate specificity. Guimbal and Kilimann (17) found that the CreaT from the electric ray, Torpedo marmorata, was 64% identical to the rabbit CreaT. Only three residues in predicted TM3 were found to be better conserved between CreaTs than other members of the neurotransmitter transporter family. One of these was a cysteine residue in TM3 that is also conserved in rabbit, rat, human, and bovine CreaTs (8, 18–21). We have investigated the importance of this residue (cysteine 144 in mammalian CreaTs) for the function of the creatine transporter.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The subcloning of a 2280-bp CDNA fragment of the bovine CreaT (GenBank access number AF027197) into the expression plasmid pCDNA3.1 (Invitrogen) has been described previously (21). The CreaT cDNA was released from pCDNA3.1–CreaT by digestion with BamHI and Clal and cut with HincII. An 822-bp 5′-fragment was inserted into pBluescript KS (Stratagene) at BamHI and HincII sites. This plasmid was used as a template for PCR-based mutagenesis (QuickChange; Stratagene). Parental wild-type DNA strands were removed by DpnI digestion, and the nicked circular DNA was used as a template for site-directed mutagenesis. Escherichia coli (DH5α) plasmid DNA was prepared as described in Ref. 22. The primers used to prepare CreaT mutants were as follows: C144S, 5′-GTTGATCGTCTTCTACTCCAACCTATTACA-3′ (forward) and 5′-TGTGTTAGGTTTGGAGTAGAAGAGC-ATCAC-3′ (reverse); C144A, 5′-AGTGTAGCTTCTCAGCAACACTTACTACA-3′ (forward) and 5′-TGTGAATGAGGTGTTGGCG-CTAGAGACCATCACCA-3′ (reverse); and C144L, 5′-TCTTCTTCTAC- TTGAAACCTTATGACT-3′ (forward) and 5′-GTAATGAGGTGTTGGCG-CTAGAGACCATCACCA-3′ (reverse). All mutant plasmid constructs were sequenced in both directions. Expression plasmids were isolated by removing BamH/HincII CreaT fragments containing the desired mutations from pBluescript and ligating them with a 3′-BamHI/ClaI CreaT cDNA fragment from pCDNA3.1–CreaT and BamHI/ClaI cut pCDNA3.1 vector.

**Transient Expression of Wild-type and Mutant CreaT** in HEK293 Cells—HEK293 cells were grown in minimum essential medium containing 10% fetal bovine serum, streptomycin, and penicillin and plated as follows: C144S, 5′-GGTGATCGTCTTCTACTCCAACCTATTACA-3′ (underlined) and the reverse 5′-GGAATTCCTAAAGAGTGCTATGGC-3′ (reverse). All mutant pBluescript constructs were sequenced in both directions. Expression plasmids were isolated by removing BamH/HincII CreaT fragments containing the desired mutations from pBluescript and ligating them with a 3′-BamH/HincII CreaT cDNA fragment from pCDNA3.1–CreaT and BamH/HincII cut pCDNA3.1 vector.

**Creatine Uptake Assay**—Cells expressing CreaT or its mutants were assayed for creatine uptake activity 48 h after transfection. The medium was removed from wells, and the cells were washed with 1 ml of prewarmed KRH uptake buffer. The KRH buffer contained 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with NaOH. The cells were incubated for 3 min in KRH at 37 °C with 1 ml of KRH containing various concentrations of [14C]creatine (American Radiolabeled Chemicals). 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. The 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 the detergent-compatible protein assay (Bio-Rad) with bovine serum albumin as standard. All assays were done in triplicate.

**The NeutrAvidin beads** were modified to determine the effect of MTS reagents on creatine uptake. Cells prepared as above were washed once with KRH before incubation for 5 min at 37 °C with various concentrations of MTSEA, MTSES, or MTSET (Toronto Research Chemicals). Creatine uptake was then assessed as described above. In protection experiments, the cells were exposed to MTSEA in the presence of creatine or its analogues. The analogues guanidinopropionate, GABA, guanidinobutyric acid, guanidinoacetae, cyclocreatine, and arginine were used at a concentration of 1 mM.

In experiments performed without Na+, KRH was prepared with N-methylglucamine in place of NaCl. To prepare KRH without Ca, NaCl, KCl, and CaCl2 were replaced with sodium gluconate, K2HPO4, and MgSO4, respectively.

**Preparation of Antibodies against the CreaT**—A 417-bp fragment encoding a 56-residue segment of the predicted carboxyl-terminal region of the bovine CreaT (GenBank access number AF027197) was synthesized by the polymerase chain reaction using the bovine CreaT cDNA as a template. The forward 5′-CCGGATCCCTGCTT-GACCGGCCAACGGGACC-3′ and the reverse 5′-GGAATTCCTAAGA-GACATTGATGACATGACCG-3′ encoded sites for HincII and BamHI (underlined), respectively, to facilitate subcloning into the vector pGEX-2T (Amersham Pharmacia Biotech). This positioned the CreaT sequence downstream and in-frame with the sequence of glutathione S-transferase. The fusion protein (GST-CreaT) was expressed in E. coli (DH5α or BL21) and purified on glutathione-agarose. Material corresponding to the intact fusion protein was purified using a 12% SDS-polyacrylamide gel and was resolved on a 10% polyacrylamide gel. The fusion proteins were exposed to MTSEA in the presence of creatine or its analogues. The analogues guanidinopropionate, GABA, guanidinobutyric acid, guanidinoacetae, cyclocreatine, and arginine were used at a concentration of 1 mM. In experiments performed without Na+, KRH was prepared with N-methylglucamine in place of NaCl. To prepare KRH without Ca, NaCl, KCl, and CaCl2 were replaced with sodium gluconate, K2HPO4, and MgSO4, respectively.

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Creatine Transporter Cys144 Involved in Substrate Binding

**RESULTS**

Creatine transporters contain a uniquely conserved cysteine (Cys144) in the third transmembrane domain, whereas other members of the Na⁺, Cl⁻–neurotransmitter transporter family have more bulky and hydrophobic side chains at this position (Fig. 1). In this study we have investigated the importance of Cys144 on the activity of the CreaT by site-directed mutagenesis and reaction with MTS derivatives.

Cys144 Is Important for the Activity of the CreaT—Variants of the CreaT were prepared in which Cys144 was replaced by Ser, Ala, or Leu. The latter residue is found in an identical position in the homologous GABA transporter (GAT-1). The activity of the C144S, C144A, and C144L mutants were 75%, 55%, and 15% of the wt CreaT (Fig. 2). The activity of all mutants was significantly above the level of endogenous creatine uptake activity of the wt CreaT (0.75 nmol/min/mg protein). The values represent the means ± S.D. of three determinations.

MTSEA Modification of Cys144—To determine whether Cys144 was the target of MTSEA modification, the C144S and C144L mutants were treated with 20 μM MTSEA for 5 min that resulted in the loss of the majority of wt CreaT activity (Fig. 6). This identifies Cys144 as the site modified by MTSEA. The C144S variant retains 70% of wt CreaT activity and is resistant to MTSEA. Thus, the C144S variant will provide a valuable background to study the structure and function of CreaT by cysteine-scanning mutagenesis.

**Creatine and Its Analogues Protect the CreaT from Inactivation by MTSEA**—Cells expressing wt CreaT were treated with
MTSEA in both the absence and presence of various concentrations of creatine. The presence of creatine was found to prevent inactivation by MTSEA in a dose-dependent manner (Fig. 7). The concentration giving 50% protection was similar to the known $K_m$ (0.2 mM) of the transporter. We also compared creatine and various creatine analogues for their ability to protect from MTSEA inactivation (Table I). Analogues that are known to be substrates of CreaT, guanidinopropionate, cyclocreatine, guanidinobutyric acid, and guanidinoacetate all reduced the inactivation by MTSEA. The degree of protection correlated with their known potency to competitively inhibit creatine transport. GABA and arginine, two molecules that are not substrates, did not protect the CreaT from inactivation by MTSEA.

**Ion Dependence of MTSEA Inactivation and Creatine Protection**

Cells expressing the CreaT were treated with MTSEA in media lacking Na$^+$ and Cl$^-$ ions. The absence of Na$^+$ or Cl$^-$ had no effect on inactivation by MTSEA (Table II). By comparison the ability of creatine to protect from MTSEA inactivation was much reduced in the absence of Na$^+$.

**DISCUSSION**

A cysteine residue (Cys$^{144}$) is found in the third transmembrane domain of all CreaTs but not other members of the Na$^+$,Cl$^-$-dependent neurotransmitter transporter family. We have found that modification of this residue with MTSEA leads to rapid inactivation of the transporter and that substrates protect from this inactivation, whereas a C144S mutant retains 70% of transporter activity. Our results are consistent with the view that Cys$^{144}$ is located in a substrate-binding site and that it is an important but not an essential determinant of creatine transport.

There are parallels between the results we have obtained for Cys$^{144}$ of the creatine transporter and those obtained by others for Cys$^{148}$ of the E. coli lactose transporter (see Ref. 27 for a review). Neither of these cysteine residues are required for creatine/Na$^+$ or lactose/H$^+$ symport. However, in each case the transporter is protected from inactivation from sulfhydryl modifying reagents by specific substrates. Replacement mutants of lac permease were used to distinguish between the possibilities that Cys$^{148}$ was either (i) a component of the substrate-binding site or (ii) far removed from the binding site with long range conformational effects providing substrate protection from in-
activation. Evidence that the size and polarity of the side chains affected the activity and specificity of the lactose transporter led to the conclusion that Cys$^{144}$ was part of a substrate-binding site (28). Similarly, we have found differences in both the initial rates and saturation kinetics of mutants in which the Cys$^{144}$ of the CreaT was replaced with Ser, Ala, or Leu. Although C144S had a $K_m$ (0.21 mM) close to that of wt CreaT (0.20 mM), the Ala mutant had a higher $K_m$ (1.77 mM), indicating a lower affinity for substrate. Both Ser and Ala are smaller than Cys and are often used interchangeably to replace cysteine in scanning mutagenesis studies. However, Ser is more hydrophilic than Ala, and this may be important if the side chain of residue 144 is in a water-accessible site. The rapid inactivation of CreaT by low concentrations of MTSEA (50 mM) suggests that Cys$^{144}$ may be present in an ionized, thiolate (RS$^-$) form. A thiolate is more likely to be present in a water-accessible site and is reported to react a billion times faster than un-ionized thiols (15). It also appears that size and hydrophobicity may decrease the affinity of the CreaT for substrate because high concentrations of GABA inhibited creatine uptake by the C144L mutant but not the wt CreaT.

The Cys$^{144}$ residue of the CreaT reacts only with MTSEA and not MTSET or MTSES derivatives. MTSET and MTSES are known to be membrane-impermeant, whereas MTSEA is a weak base and may cross the membrane (15). MTSET and

\[ \text{MTSEA} + \text{CreaT} \rightarrow \text{MTSEA-CreaT} \]

\[ \text{MTSES} + \text{CreaT} \rightarrow \text{MTSES-CreaT} \]

\[ \text{MTSET} + \text{CreaT} \rightarrow \text{MTSET-CreaT} \]

The Cys$^{144}$ residue is in an ionized, thiolate form and is unlikely to react with MTSET or MTSES derivatives. MTSET and MTSES are known to be membrane-impermeant, whereas MTSEA is a weak base and may cross the membrane (15). MTSET and

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MTSES are also larger than MTSEA and contain bulky charged groups that may prevent them entering a substrate translocation pathway for creatine. The rapid reaction with low concentrations of MTSEA and the ability of creatine to protect from inactivation suggest Cys\textsuperscript{144} to be located in a water-accessible site. However, it is difficult to determine whether MTSEA enters this site from the extracellular or cytoplasmic side of the membrane. Creatine appears to protect from MTSEA inactivation by being transported, because protection is significantly reduced by the absence of Na\textsuperscript{+} or Cl\textsuperscript{−}. We thus favor a view in which Cys\textsuperscript{144} is accessible from the extracellular side of the membrane. Na\textsuperscript{+} or Cl\textsuperscript{−} are not required for MTSEA inactivation, so it appears unlikely that a conformational change is induced by these ions.

A close correlation was found for the concentration of creatine required for 50% protection and the experimentally determined \( K_m \) for creatine transport (~0.2 mM). Also, the rank order of potency of creatine analogues that protect from MTSEA (guanidinopropionate > creatine > cyclocreatine > guanidinobutyric acid > guanidinoacetate) is in accordance with known affinities of these substrates for the transporter. Furthermore, only substrates of the CreaT could protect from MTSEA inactivation. No protection was obtained with GABA or arginine, derivatives that contain functional groups present in creatine but are not competitive substrates of CreaT. These data are consistent with the view that Cys\textsuperscript{144} is close to or part of the creatine-binding site of the CreaT.

The identification of a residue within the third transmembrane domain of the CreaT as part of a substrate binding site is consistent with research on other members of the Na\textsuperscript{+},Cl\textsuperscript{−}-dependent neurotransmitter transporter family. Tyr\textsuperscript{140} in TM3 of GAT-1 has been found to be critical for substrate recognition (13). All members of this transporter superfamily contain a Tyr in an equivalent position, and Biamuth \textit{et al.} (13) suggested a role for this residue in binding an amino-group. A mutant form of the SerT in which this Tyr was replaced with a cysteine had reduced serotonin and cocaine binding (14). It was suggested that Ile\textsuperscript{172} and Ile\textsuperscript{179} of SerT are part of a serotonin- and cocaine-binding site. Further studies from this group led to the conclusion that TM3 constitutes part of a permeation pathway for both SerT and norepinephrine transporter (16). Cys\textsuperscript{144} of the CreaT and Ile\textsuperscript{172} of the SerT both occupy equivalent positions within the predicted helices of TM3 (Fig. 1 and Refs. 16 and 17). The cysteine residues in wild-type CreaT and the I172C SerT mutant react with MTS reagents, and in each case the substrate protects from inactivation. The present work with the naturally occurring cysteine at position 144 of the CreaT strengthens the importance of this region of TM3 for substrate binding by Na\textsuperscript{+},Cl\textsuperscript{−}-dependent neurotransmitter transporters.

Cysteine-scanning mutagenesis is a powerful technique when combined with hydrophilic MTS reagents to study structure-function relationships and the topology of membrane proteins. The ideal starting point is a cysteine-less protein. This has been possible for only a few membrane proteins, the lactose permease, the Nha-Na\textsuperscript{+/H\textsuperscript{+}} transporter, and the glutamate transporter (17, 29, 30). The CreaT contains 22 cysteine residues, several of which are conserved in the related GABA and SerT and are likely to be involved in disulfide bonds. It appears that production of a cysteine-less CreaT that retains function would be very unlikely. We have found the C144S mutant of the creatine transporter to retain ~70% of the activity of wt CreaT and be resistant to MTS reagents. This mutant will provide an excellent background for probing residues in TM3 and other interesting domains of the CreaT by cysteine-scanning mutagenesis.
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