An Arginine to Glutamine Mutation in Residue 109 of Human Ornithine Transcarbamylase Completely Abolishes Enzymatic Activity in Cos1 Cells

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Abstract

Ornithine transcarbamylase (OTC) is an important enzyme in the detoxification of ammonia to urea, and its deficiency is the most common inborn error of ureagenesis in humans. Among 24 cases of OTC deficiency previously examined, three unrelated individuals all showed loss of a Taq I site in the *OTC* gene corresponding to codon 109, suggesting that this Taq I site may be prone to mutation. Two of these patients demonstrated the same C → T transition (in antisense strand) converting Arg109 to Gln. Although these studies implied a strong association between the missense mutation and OTC-deficient phenotype, a causal relationship could not be firmly established. We have investigated this relationship by reconstructing the mutation in vitro. A full-length human OTC cDNA was cloned into an SV40-based expression vector and has been reproducibly expressed at high levels in the cell line Cos1. By site-directed mutagenesis of this wild type sequence, we constructed a missense mutation which contains the C → T transition. Electroporation and transient assay in Cos1 indicated that the specific activity of mutant OTC was 100-fold lower than that of wild type. This result confirms that the Taq I alteration leading to the Gln missense is responsible for the OTC deficiency affecting the above patients.

Introduction

Ornithine transcarbamylase (OTC)[1] is a urea cycle enzyme which condenses carbamyl phosphate and ornithine to form citrulline. A homotrimer of 36-kD subunits, OTC is localized to the mitochondrial matrix and is found exclusively in two tissues: the liver and small intestine. With the recent cloning of the human *OTC* gene, the gene was found to be a 73-kb gene containing 10 exons (1).

An X-linked disease, a deficiency of OTC is the most common inborn error of ureagenesis in humans and is a disease with frequent new mutations (2, 3). Over 100 cases of OTC deficiency have been examined and documented. This condition results in the inability to detoxify ammonia and ranges in severity from neonatal lethality to mild hyperammonemia with developmental delay. It is believed that the severity of symptoms correlates with the nature of the molecular defect (4–7). Biochemical studies on liver biopsy samples have indicated that the disease can result from either quantitative changes (decreased production of OTC) or qualitative changes (altered ligand affinity or catalytic potential) in the enzyme. Unfortunately, few attempts have been made to correlate structural defects with clinical (functional) defect.

With the availability of cDNA probes for OTC, examination of OTC^- individuals has shifted to the DNA level. Abnormalities have been found to range from gross gene deletions (8–11) to small mutations detected only with restriction fragment length alterations (2, 3). We had previously observed that point mutations constitute a class of OTC mutations that are not only variable in location but also recurrent in independent families (2, 3). Among 24 cases examined, three unrelated individuals all showed loss of the same Taq I site corresponding to codon 109. Furthermore, two of these patients demonstrated the same C → T transition (antisense), converting Arg109 to Gln. Although strongly suggestive, these observations could not firmly establish a causal relationship between the missense mutation and OTC deficiency. The impact of this mutation is of particular interest because it is generally believed that Arg109 does not reside in either the ornithine- or the carbamyl-phosphate–binding sites. In an attempt to understand molecular structure in relationship to the spectrum of clinical disease, we have reconstructed the Arg to Gln mutation in vitro by site-directed mutagenesis and have subsequently shown that the missense mutation lacks any OTC activity when expressed in tissue culture.

Methods

Molecular biology

All techniques in recombinant DNA technology were performed according to Maniatis et al. (12).

Plasmids and oligonucleotide

The oligonucleotide used in mutagenesis is an 18-mer containing the sequence TATACACT<u>T</u>GAGCCAATA. It is the antisense sequence derived from basepair 317–334 of the published OTC cDNA sequence (13) but contains the C → T transition at basepair 325 converting Arg to Gln (underlined nucleotide).

pHOC3 contains a 1.5-kb OTC insert originally isolated from a human liver cDNA λgt11 library (2). pSV2a-dHFR is a ColE1 plasmid containing an SV40 origin of replication and SV40 early promoter and polyadenylation site flanking the dihydrofolate reductase cDNA (14). pSV2-PAP is the placental alkaline phosphatase gene cloned into the above pSV40 backbone (15).

Site-directed mutagenesis and DNA sequencing

Plasmids, reagents, and protocol used in the mutagenesis were all obtained from Bio-Rad Laboratories, Richmond, CA (Mutagene M13 in

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1. *Abbreviations used in this paper:* OTC, ornithine transcarbamylase; PAP, placental alkaline phosphatase.

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vitro mutagenesis kit). The oligonucleotide used is described above. DNA sequencing was performed using the new Sequenase technology and ^{35}S-labeled adenine nucleotides from standard M13 primers from New England Biolabs, Beverly, MA.

DNA transfection for transient expression

Cos1 is an immortalized African green monkey cell line which is permissive for SV40 replication (16) and was used for all DNA transfections. DNAs were introduced by electroporation for transient assay. The electroporation protocol was modified from Chu et al. (17). Cells were grown to 70% confluence T-75 flasks in DME/10% FCS, washed in 1× PBS, harvested by trypsin digestion, and resuspended in 1× HBSS at a concentration of 5×10^5. 80 μg of OTC DNA and 5 μg of placental alkaline phosphatase (PAP) DNA were added to each cuvette. Electroporation was done in 1-ml cuvettes with platinum electrodes at 175 V. After a 3-d incubation period in DME/10% FCS, cells were prepared for enzymatic assays as described below.

Enzymatic assays for OTC and PAP

After transient expression, cells were harvested by scraping with rubber policemen, washing in 1× PBS, resuspending in 1 ml 1× PBS, and aliquoted as follows: 200 μl for PAP assay, 700 μl for OTC assay, and 100 μl for Bradford assay. Each enzymatic assay is a colorimetric assay performed as described below.

PAP assay (15). 200 μl of cells were spun down and resuspended in 20 μl of 50 mM Tris–pH 7.5, 1 mM $MgCl_2$. 10 μl of cells were heated to 65°C for 30 min, added to 5 mM *p*-nitro-phenyl phosphate substrate reaction mixture, and incubated for 30 min at 30°C. PAP activity was then determined from spectrophotometric measurements at 405 nm.

OTC assay (18). 700 μl of cells microfuged and resuspended in 200 μl of mitochondrial lysis buffer (0.5% Triton, 10 mM Hepes, 2 mM DTT). Lysis was done by sonication in the W375 Ultrasonic Processor (Heat Systems, Ultrasonics, Inc., Farmingdale, NY) at maximal power for 2 min at 4°C, and then incubated on ice for 30 min. 50 μl of homogenate was then added to a solution of ornithine and triethanolamine to a final volume of 675 μl. OTC reactions were begun by adding carbamyl phosphate (75 μl of 150 mM stock). Final concentrations of each reagent were: 5 mM ornithine, 15 mM carbamyl phosphate, and 270 mM triethanolamine. Reactions were stopped by 375 μl of 3:1 phosphoric acid/sulfuric acid (by volume). Citrulline production was then determined by adding 47 μl 3% 2,3-butanedionemonoxime, boiling in the dark for 15 min, and reading at A-490 nm. The positive control used in these OTC assays is a 2-μl sample of rat liver mitochondrial homogenate as described in (18).

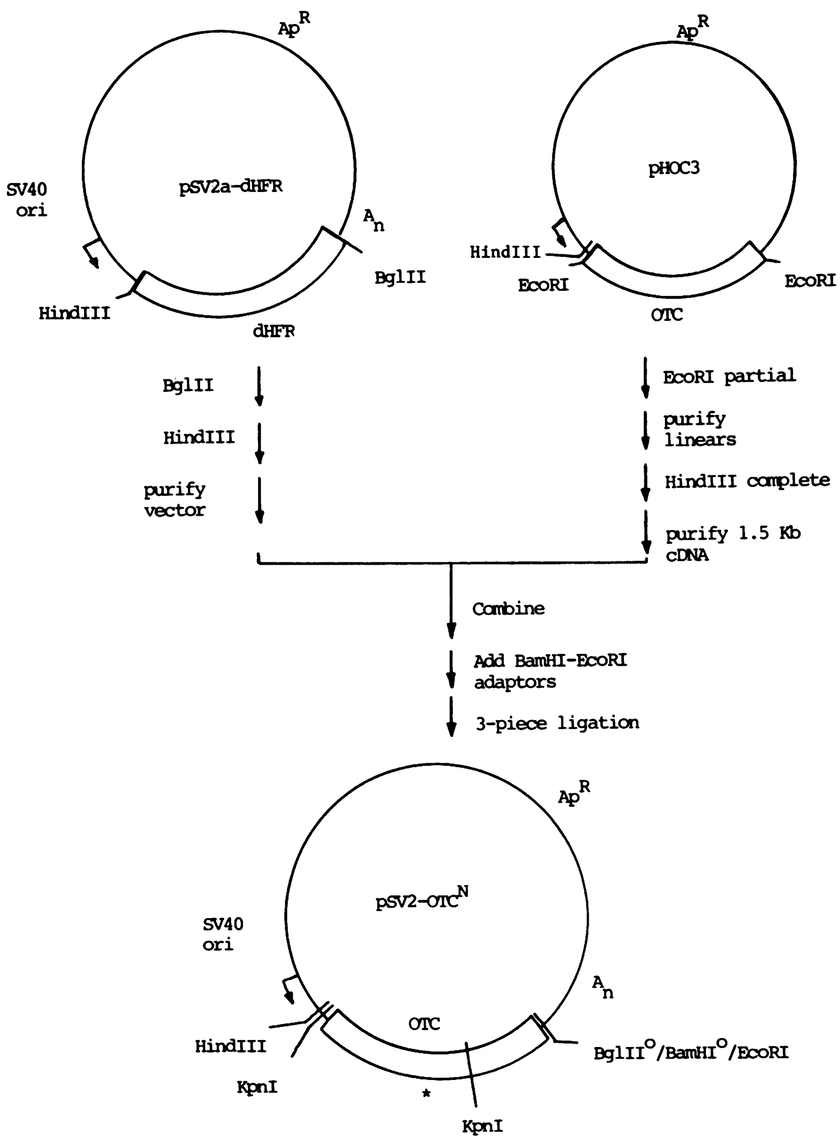

Figure 1. Cloning of OTC cDNA into an SV40-based expression vector. The OTC cDNA was excised from pHOC3, a clone derived from the human liver λgt11 library (2). pHOC3 was partially digested with Eco RI. Linearized species were electrophoresed into an agarose gel, isolated on NA45 paper, and then digested with Hind III. The complete OTC cDNA could be identified as a 1.5-kb fragment and was then purified by gel electrophoresis. The pSV2a vector was prepared by excising the dHFR gene from the parent pSV2a-dHFR by digesting with Bgl II and Hind III and purifying the vector backbone. Then the vector and cDNA were combined with Bam HI–Eco RI adaptors in a three-piece ligation reaction to create pSV2-OTC^N. The pSV2a vector is derived from pBR322 and SV40. It contains ColE1 origin of replication, the resistance marker for ampicillin, the SV40 origin of replication, early promoter, and the polyadenylation site. ↳ designates the transcriptional start site of the SV40 early promoter, A_n marks the polyadenylation site, Bgl II^0/Bam HI^0 indicates destroyed restriction sites, and * refers to the position of Arg109.

Bradford protein analysis.
Bradford protein analysis was performed as recommended by Bio-Rad Laboratories. A standard curve was generated by using linearly increasing concentrations of BSA.

Results

Cloning of OTC cDNA into SV40-based expression vector. To maximize expression of our DNA constructs, we used the expression vector pSV2a containing an SV40 origin of replication (14), enabling electroporated DNA to be amplified as extrachromosomal elements in the permissive cell line Cos1. The scheme used to clone wild type *OTC* into pSV2a is depicted in Fig. 1. The *OTC* gene was excised from pHOC3, a cDNA clone derived from the human liver λgt11 library (2). pHOC3 was partially digested with Eco RI. Linearized species were electrophoresed into an agarose gel, isolated on NA45 paper, and then digested with Hind III. The complete OTC cDNA could be identified as a 1.5-kb fragment and was then purified by gel electrophoresis. The pSV2a vector was prepared by excising the dHFR gene from the parent pSV2a-dHFR by digesting with Bgl II and Hind III and purifying the vector backbone. Then the vector and cDNA were combined with Bam HI–Eco RI adaptors in a three-piece ligation reaction to create pSV2-OTC^N.

pSV2-OTC^N was then electroporated into Cos1 cells for transient expression. Subsequent OTC assays revealed that this construct reproducibly expressed high levels of OTC in tissue culture (Table I).

Oligonucleotide-directed mutagenesis. An 850-bp Kpn I fragment spanning the desired mutation site was cloned into M13 at the Kpn I site. Site-directed mutagenesis was performed with the Mutagene Kit (Bio-Rad Laboratories) and using the 18-mer oligonucleotide containing the single basepair change corresponding to the Arg109-to-Gln mutation. Mutants were initially screened by the loss of a unique Xho I site (which includes the four-base Taq I recognition sequence). Subsequent nucleotide sequence analysis confirmed that the C → T transition is the only change present in the mutant OTC. Finally, the mutagenized Kpn I fragment was cloned back into the wild type pSV2-OTC background for expression in vivo. Restriction analyses demonstrated the integrity of the Kpn I sites and retention of other landmark sites. This mutant construct is designated pSV2-OTC9.

Comparative activities of wild type and mutant OTC. Mutant or wild type pSV2-OTC was electroporated into Cos1 cells for transient expression. pSV2-PAP was cotransfected with the OTC cDNAs as an internal control for transfection efficiency. After a 3-d incubation period, cells were harvested for OTC, PAP, and Bradford assays. Table I shows that the transfection efficiencies of each sample were comparable, as determined from the PAP activities. The wild type OTC (pSV2-OTC^N) has a high specific activity of 80.83 μM/min per mg. In contrast, the mutant OTC (pSV2-OTC9) has a 100-fold lower specific activity of 0.94 μM/min per mg. Since this level is no higher than that of background (pSV2-PAP), we conclude that the Arg-to-Gln mutation completely abolishes OTC activity.

Table I. Relative Enzymatic Activities of Mutant and Wild-Type OTCs Indicate that Arg109 Mutation Completely Abolishes OTC Activity in Cos1

	OTC activity	PAP activity	Specific activity
	OD_{490}/h	*OD_{595}/h*	*μM/min per mg*
Liver extract	4.400	—	20.63
pSV2-PAP	0.090	1.300	0.95
pSV2-OTC^N	5.160	1.456	80.83
pSV2-OTC9	0.078	1.480	0.94

pSV2-OTC^N contains wild-type OTC cDNA, whereas pSV2-OTC9 contains the missense cDNA (Arg109 → Gln). pSV2-PAP was used as the negative control for background activity, and rat liver mitochondrial homogenate was used as positive control for OTC activity. Cos1 cells were electroporated with 80 μg OTC cDNA and/or 5 μg PAP cDNA and then harvested on the third day of transient expression for enzymatic assays. OTC and PAP activities are defined as the amount of colored product formed per hour, as determined spectrophotometrically. These figures were calculated for 10% of total culture. Specific activity is defined as micromolar citrulline formed per minute per milligram of total protein, where OD_{490} of 1.0 is equivalent to 5.5 mM of citrulline. The specific activity was not formally normalized for transfection efficiency since our PAP activities show that transfection efficiencies varied little.

Discussion

We have recreated the Arg109 → Gln OTC mutation in vitro and have shown that the missense mutation completely abolishes OTC activity in Cos1 cells. This result confirms that the C → T transition in the two patients bearing the altered Taq I site is indeed responsible for their OTC-deficient phenotype.

The biochemical impact of this C → T transition is unclear. Preliminary analysis of mRNA from cells expressing the mutant OTC cDNA driven by the SV40 promoter shows that the basepair alteration does not affect steady state OTC mRNA levels or size. However, no introns are present in this artificial expression vector construct and therefore the effect of the mutation on processing, particularly splicing, of transcripts made from an endogenous cellular *OTC* gene cannot be assessed. However, the fact that a protein carrying this mutation shows complete loss of enzyme activity suggests a critical role for this residue in the protein without having to invoke an abnormality in splicing as well. In contrast, spf^{ash}, a missense mutation at a different arginine residue, Arg129 of mouse OTC, occurs at an intron-exon boundary and exerts its deleterious effect by unmasking a cryptic splice site (19). The new splice site is used 95% of the time and leads to the production of a nonfunctional protein. However, when the correct splice site is used, a properly spliced mRNA is translated to a functional protein that contains the amino acid substitution. The spf^{ash} mutation is therefore strictly a pretranslational mutation which has little or no effect on the protein product.

In light of our preliminary Northern analysis, we have shifted our attention to the analyses of protein stability, assembly, and function.

Interestingly, Arg109 is conserved across the spectrum of bacterial, yeast, and mammalian OTCs (20). Furthermore, it is conserved in *Escherichia coli* aspartate carbamyl transferase, an enzyme condensing carbamyl phosphate and aspartate that is related to OTC. Sequence comparisons among OTCs of different species have demonstrated that the decapeptide comprising residues 53–62 is highly conserved (21–24). The core sequence ser-thr-arg-thr-arg contained within the decapeptide has been shown to bind phosphates in several other proteins (25), and has been demonstrated to be highly reactive in bovine OTC (26). Thus, it is speculated that this region of OTC may be involved in binding carbamyl phosphate. Similarly by sequence comparison, the hexapeptide phe-leu-his-cys-leu-pro (residues 268–273) appears to be a highly conserved sequence among all OTCs examined. It has been shown that chemical modification of this conserved cysteine residue in bovine and bacterial OTCs by 5,5′-dithiobis (2-nitrobenzoic acid) results in total loss of affinity for ornithine (27–29). This hexapeptide is therefore believed to reside in the site of ornithine binding.

Although the highly conserved Arg109 is contiguous with neither the concensus decapeptide nor the hexapeptide, one cannot exclude the possibility that the residue could be at either active site in the three-dimensional protein. The effect of Arg109 could thus be a loss of catalytic potential caused by the inability to bind ligand. Whether the mutant protein could bind either substrate will be studied by affinity chromatography using ligand-coupled matrices. X-ray crystallography and photoaffinity-labeling experiments would also be needed to clarify these points.

A second possible effect of the missense mutation may be on trimer assembly. There is, as yet, no data on sequence requirements for trimer formation. Our approach to determine what effect Arg109 has on protein assembly will involve immunoblot analysis of native vs. denatured proteins. Finally, there are the possibilities that the mutation could affect protein conformation and/or turnover. Whatever the structural effect, this naturally occurring mutation illustrates the lethality of altering a residue that has been so rigidly preserved by evolution. Finding the molecular role of this residue would further our understanding of OTC enzymology.

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