

Structural aspects of the p.P222Q homozygous mutation of *HSD3B2* gene in a patient with congenital adrenal hyperplasia

Aspectos estruturais da mutação homocigótica p.P222Q do gene *HSD3B2* em um paciente com hiperplasia congênita da adrenal

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SUMMARY

Type II 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD2), encoded by the *HSD3B2* gene, is a key enzyme involved in the biosynthesis of all the classes of steroid hormones. Deleterious mutations in the *HSD3B2* gene cause the classical deficiency of 3 β -HSD2, which is a rare autosomal recessive disease that leads to congenital adrenal hyperplasia (CAH). CAH is the most frequent cause of ambiguous genitalia and adrenal insufficiency in newborn infants with variable degrees of salt losing. Here we report the molecular and structural analysis of the *HSD3B2* gene in a 46,XY child, who was born from consanguineous parents, and presented with ambiguous genitalia and salt losing. The patient carries a homozygous nucleotide c.665C>A change in exon 4 that putatively substitutes the proline at codon 222 for glutamine. Molecular homology modeling of normal and mutant 3 β -HSD2 enzymes emphasizes codon 222 as an important residue for the folding pattern of the enzyme and validates a suitable model for analysis of new mutations. Arq Bras Endocrinol Metab. 2010;54(8):768-74

SUMÁRIO

A enzima 3 β -hidroxisteroide desidrogenase/ Δ^5 - Δ^4 -isomerase do tipo 2 (3 β -HSD2), codificada pelo gene *HSD3B2*, é importante na biossíntese de todas as classes de hormônios esteroides. As mutações no gene *HSD3B2* podem causar deficiência da 3 β -HSD2 da forma clássica. É de herança autossômica recessiva e uma das causas mais raras de hiperplasia congênita da adrenal (HCA). A deficiência dessa enzima leva frequentemente à ambiguidade genital e à insuficiência da adrenal em recém-nascidos com vários níveis de perda de sal. Neste trabalho, foi feito o estudo estrutural e molecular do gene *HSD3B2* em um paciente 46,XY, filho de pais consanguíneos, com ambiguidade genital e perda de sal. O paciente é homocigoto para a troca nucleotídica c.665C>A no éxon 4, que putativamente leva à substituição de uma prolina do códon 222 por uma glutamina. A modelagem molecular por homologia das enzimas 3 β -HSD2 normal e mutantes ressaltou que a prolina no códon 222 é um resíduo importante no enovelamento da enzima e validou um modelo adequado para avaliações de novas mutações. Arq Bras Endocrinol Metab. 2010;54(8):768-74

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INTRODUCTION

The steroidogenic enzyme 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD) is a membrane-bound NADH-dependent enzyme involved in the biosynthesis of all the classes of steroid hormones,

namely glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens (1). Those steroid hormones play an important role in processes such as differentiation, development, and growth and in physiological functions in the most human tissues (2).

The dehydroepiandrosterone (DHEA) is used as substrate in a two-step reaction catalyzed by 3 β -HSD. During the reaction, the reduction of NAD⁺ to NADH occurs by a rate-limiting activity of 3 β -HSD followed by the NADH recruitment for the activation of isomerase activity on the same enzyme. A previous model of the type I enzyme suggests that both 3 β -HSD and isomerase domains of the enzyme are linked by a shared coenzyme domain that may function as both binding site domain for NAD⁺ during the 3 β -HSD reaction and as coenzyme domain for the allosteric activation for isomerase activity (3).

In humans, there are two types of 3 β -HSD isoenzymes encoded by two genes that are very similar in structure and both are located on 1p13.1 (2,4,5). Type I gene (*HSD3B1*) is expressed in placenta and peripheral tissues. Type II gene (*HSD3B2*) encodes the adrenal and gonadal 3 β -HSD enzymes (5,6). In the adrenal, type II 3 β -HSD is a key enzyme required for the production of cortisol and aldosterone (7).

Deleterious mutations in the *HSD3B2* gene cause classical 3 β -HSD2 deficiency (OMIM # +201810), which is an autosomal recessive inherited form of congenital adrenal hyperplasia (CAH) that impairs steroidogenesis in both the adrenals and gonads (8-11). The clinical manifestation of classical 3 β -HSD2 deficiency ranges from salt-losing to non salt-losing forms in both sexes. In newborns, 3 β -HSD2 deficiency results in ambiguity of the external genitalia in genetic males, while affected females exhibit normal sexual differentiation or partial virilization. During adolescence, 3 β -HSD2 deficiency results in variable degrees of hypogonadism in boys and hyperandrogenism (premature pubarche and hirsutism) in girls. The nonclassical form of 3 β -HSD2 deficiency has been described in females with hyperandrogenism, but no *HSD3B2* mutations were found in those patients (12-18).

In the present study we describe the clinical and molecular characterization of a patient diagnosed as having the classical form of congenital adrenal hyperplasia (CAH) due to 3 β -HSD2 deficiency. Molecular analysis of the *HSD3B2* gene revealed the homozygous c.665C>A missense mutation in codon 222 changing the proline to glutamine. Molecular homology modeling of 3 β -HSD2 was performed. We used sequences of three-dimensional structure determined by X-ray crystallography that showed 30% identity with the protein sequence of 3 β -HSD2. The proposed model empha-

sizes the critical importance of the P222 residue on the overall 3 β -HSD2 enzyme integrity and activity.

CASE REPORT

The patient is a Caucasian male child born at term after an uneventful pregnancy from a consanguineous marriage. At birth he weighed 3.2 kg and his height was 50 cm. He was referred to us for investigation of ambiguous genitalia. In the first evaluation, at 23 days of life, his height was 50 cm, weight was 2.85 kg; he had a 3 cm-long phallus and complete fusion of the labioscrotal folds with only one palpable gonad. His karyotype was 46,XY. He had high serum levels of 17OHPregesterone (942 ng/mL – 28.5 nmol/L) with normal dosages of sodium (137 mEq/L) and potassium (6.0 mEq/L). Thus, diagnosis of CAH was suspected. Despite dexamethasone treatment he presented poor weight gain with a hyponatremic (129 mEq/L) dehydration episode associated with hyperkalemia (7.6 mEq/L) at 2 months of age. Laboratory investigation showed a DHEA/ Δ^4 -DIONE ratio of 17.5. Diagnosis of CAH due to 3 β -HSD deficiency was considered. Thereafter, therapy with glucocorticoid (hydrocortisone) and mineralocorticoid (fludrocortisone) replacement was established leading to gain of weight and normal growth rate.

This study was approved by the appropriate Ethics Committee from the State University of Campinas (São Paulo, Brazil) and informed consents were obtained from the patient and his parents.

METHODS

Genomic DNA was isolated from blood leukocytes using standard techniques. Primers and conditions for PCR amplification of the entire coding region of the *HSD3B2* gene were based on a published report (6). Purified PCR products were sequenced in both sense and antisense orientations using ABI PRISM 377 Automated DNA Sequencer according to the manufacturer's recommendations (Applied Biosystems, USA).

The three-dimensional structure of both mutant and native 3 β -HSD2 proteins were modeled using the crystal structure of Vestitone Reductase from *Medicago sativa* L. (Protein Data Bank accession number 2P4H) as a template (19). Models were created and validated by using default settings and parameters of the Swiss

Model web-served software. The modeled protein structures were produced and analyzed by using the web-based BlueStar STING software (20).

RESULTS

DNA sequencing of the patient's *HSD3B2* gene revealed the homozygous missense mutation p.P222Q (CCA>CAA). Both parents and his sister were found to be heterozygous for this mutation (Figure 1A). The nucleotide substitution was confirmed by at least three independent PCR and sequencing analyses, in both sense and antisense strands. Protein alignment showed that the proline at residue 222 is highly conserved among the mammalian 3 β -HSD family, as demonstrated in figure 1B.

Structural studies were carried out with both p.P222Q and p.P222T mutations for comparison purposes. Blast results for the native human 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase showed the

vestitone reductase from *Medicago sativa L.* (PDB-ID 2P4H) as the most similar structure with a score of 31%. Based on this sequence identity, a three-dimensional ribbon model of human 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase was constructed using the vestitone reductase sequence as template (Figure 2A). The ribbon structures for both native and for the two different 3 β -HSD2 mutants in residue 222 demonstrated a prominent side-chain group for both glutamine and threonine as compared to the native proline (Figure 2B and C). In addition, the analysis of internal contacts (Figure 2D, E, and F) showed that the native residue Pro²²² makes a hydrogen-bond with Y254 in main chain and hydrophobic interactions with Y224, V220, and also with Y254. The mutated residues Gln²²² and Thr²²² eliminate the hydrophobic interaction with Val²²⁰. In complement, T222 enzyme interacts with residue Y254 by establishing hydrogen-bonds with the main chain and side chains. The domain formed

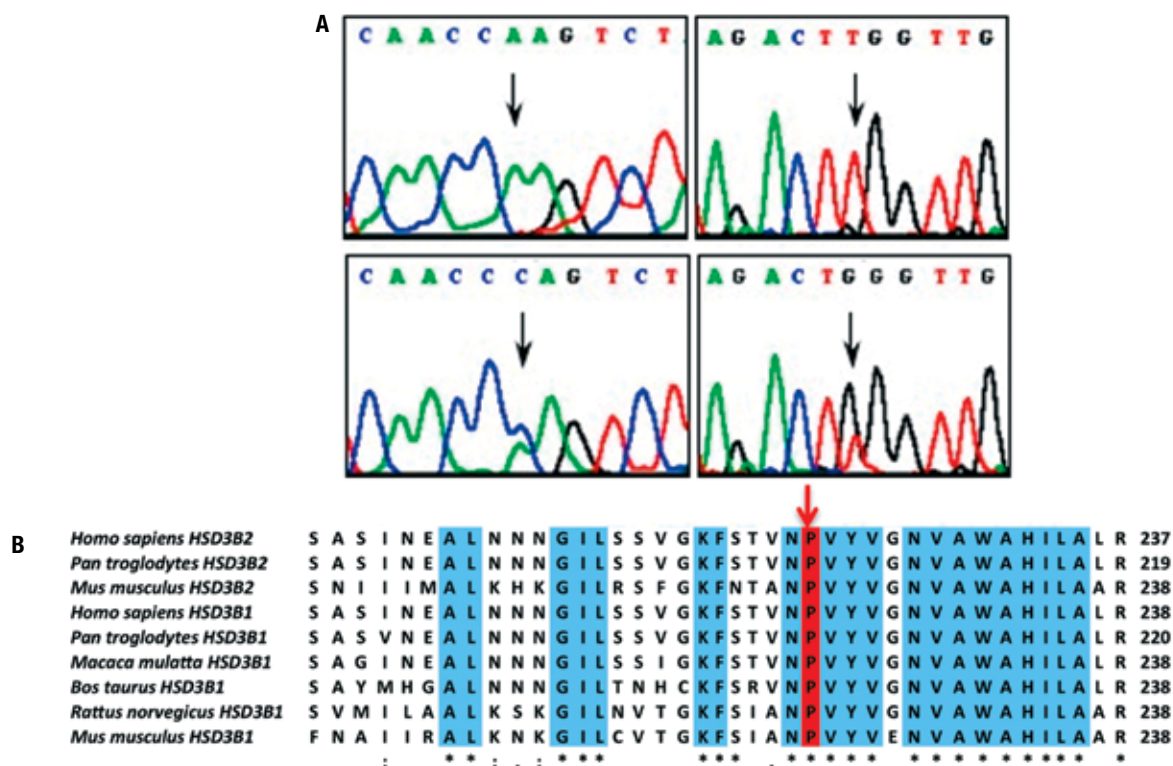


Figure 1. (A) Partial nucleotide sequence of exon 4 of the *HSD3B2* gene showing the C>A transversion (black arrows) in both sense (left) and antisense (right) strands. Upper panels show patient's sequence who is homozygous for the nucleotide change; lower panels show his sister's sequence who is heterozygous. **(B)** Clustalw comparison of the partial amino acid sequences of mammalian 3 β -HSD isoenzymes. Residue Pro²²² is marked in red (red arrow). Amino acid residues are designated by single letter code and numbered according to the first NH₂-terminal methionine of human 3 β -HSD2. Conserved residues in 3 β -HSD2 proteins are boxed in blue.

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by residues 251-274, which is a region for substrate binding, demonstrates important structural modifications when P222 is replaced by Q222 or T222. Most

remarkable is that the wild-type protein presents a flexible turn while both mutant proteins show a β -sheet in this region (Figure 3).

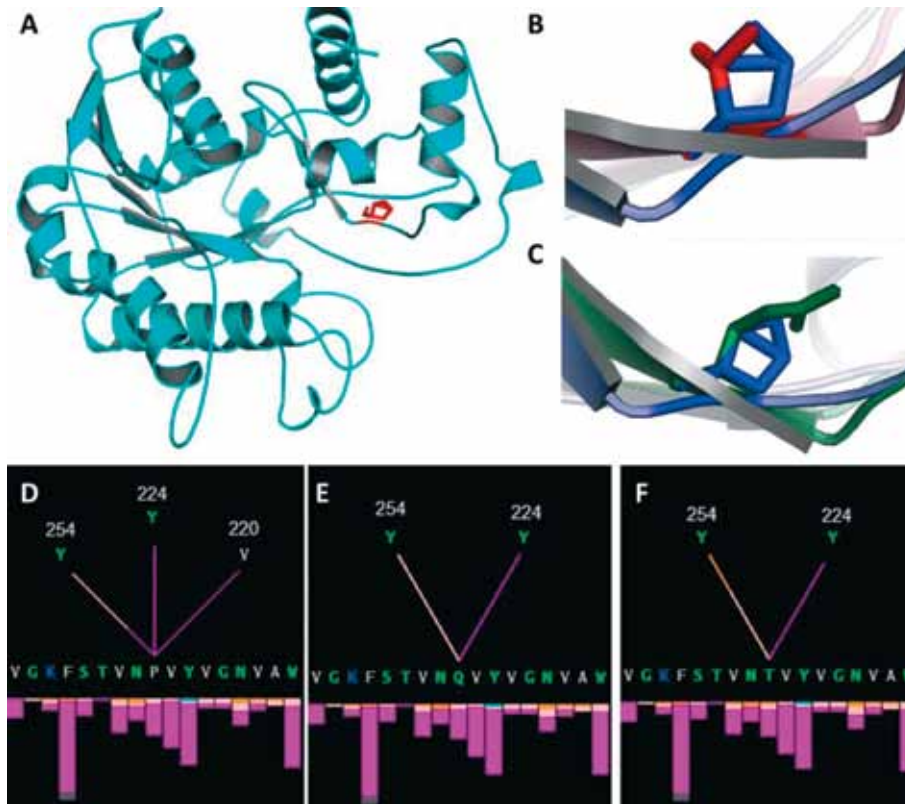


Figure 2. (A) Ribbon structure of native 3β -HSD2 (cyan), the P222 residue is denoted in red. (B) Aligned ribbon structure of both native and mutants 3β -HSD2 demonstrating the prominent side-chain group of the mutant threonine (red) as compared to the side-chain group of proline (blue). (C) P222 on the native enzyme (blue) and Q222 mutant enzyme (green). STING report contacts obtained from the BlueStar STING software. The images show internal contacts of proline, glutamine, and threonine on the respective native (D), Q222 (E) and T222 (F) enzymes. Lines denote different interactions: hydrophobic interactions (magenta); hydrogen bond main chain-main chain (rose); hydrogen bond side chain-main chain (salmon). Bars below each amino acid are internal contacts in each amino acid. Bar color represents the contact type and its width represents the contact number.

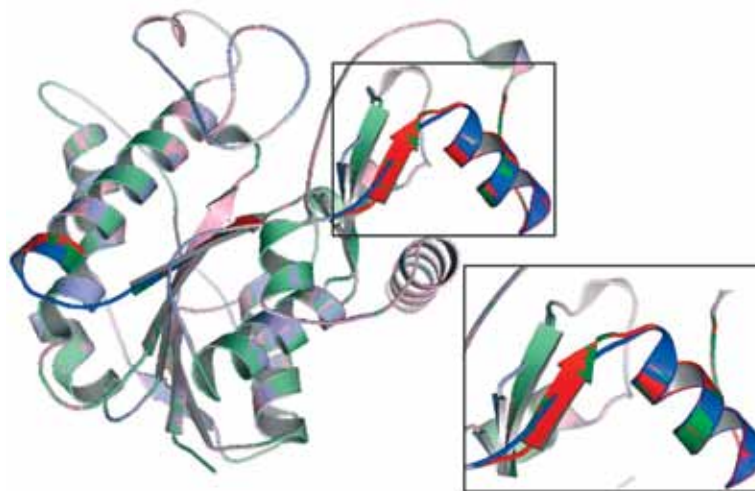


Figure 3. Overlap of wild-type structure of 3β -HSD2 (cyan) and the Q222 (magenta) and T222 (green) mutant proteins. Substrate binding domains are identified in different colors: wild-type (blue), Q222 (red), and T222 (green). In detail, region 251-274 substrate binding domain.

DISCUSSION

p.P222Q mutation has already been described as leading to a complete loss of 3β -HSD2 activity (21). The severe form of 3β -HSD2 deficiency in the homozygous patient here reported confirms a good correlation genotype-phenotype for the mutation. These findings suggest that residue position 222 is important for enzymatic activity.

Two different *missense* mutations have been identified in codon 222, depending on the mutated nucleotide being located in the first or in the second nucleotide of the codon (21-23). The c.664C>A transversion in the first nucleotide of codon 222 (CCA>ACA) leads to the replacement of a proline by a threonine. The *missense* mutation p.P222T has been identified in an Eastern European female neonate with classic salt-losing disorder (23). Whereas, the transversion c.664C>A in the second nucleotide of codon 222 CCA>CAA converts a proline to a glutamine. The *missense* mutation p.P222Q has been previously identified in two Algerian siblings (22). In the Brazilian population the p.P222Q mutation was found in two sisters in compound heterozygosis with the p.G129R mutation (24) and in a homozygous male patient with the salt-losing form of 3β -HSD2 deficiency (25). The case described here is a Brazilian male with typical clinical features of classical 3β -HSD2 deficiency who is also homozygous for the p.P222Q mutation.

Both mutations on P222 residue abolished the 3β -HSD2 activity as demonstrated by *in vitro* assays (22,23). The importance of this residue is evidenced by the fact that the residue is predicted to be within the membrane-spanning domain suggesting a putative role on the ultimate folding pattern of the enzyme (2). In addition, P222 residue is located adjacent to the substrate-binding domain being highly conserved in that particular position (26). Although both p.P222Q and p.P222T mutations render the 3β -HSD2 enzyme with no detectable activity from the structural point of view, they probably do so due to different effects. Moisan and cols. (22) report that p.P222Q enzyme did not show any evidence of protein instability. They discuss that the absence of enzymatic activity was probably due to alterations in the catalytic activity of the enzyme, such as significant changes in the substrate-binding domain introduced by the presence of glutamine in codon 222. On the other hand, p.P222T enzyme showed no detectable signal on Western blot despite mRNA pro-

duction, suggesting a totally unstable protein (23). It was considered that the severe instability of the enzyme was the main detrimental mechanism that profoundly decreased 3β -HSD2 activity (23). In order to obtain novel information concerning the structure-function relationship of 3β -HSD2 mutant enzymes we characterized the functional significance of p.P222Q and p.P222T amino acid replacement in enzyme activity by molecular modeling the p.P222Q and p.P222T enzymes. To produce a three-dimensional model useful for interpreting biochemical data and for proposing and testing mechanisms of action for the 3β -HSD2 protein a model should be obtained based upon a protein with an already resolved crystallographic structure. To select such a model, one parameter was that the fold of the proteins should be similar enough so that energy of the model would be minimal (27-29). To establish similarities among candidate sequences the blast algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used. The three-dimensional structure of human type 1 3β -HSD/isomerase was modeled before using the crystal structure of UDP-galactose 4-epimerase from *E. coli* as template, an enzyme with 30% overall sequence identity (30). Here we tested the vestitone reductase sequence from *Medicago sativa* L. as template for a model of human type 2 3β -HSD/isomerase to analyze the effect of mutations in the protein sequence.

The consequence of amino acid replacement on 3β -HSD2 activity can be inferred according to the biochemical properties of each amino acid. Proline has a non-polar side-chain and is hydrophobic, tending to cluster with other hydrophobic residues on the inside of the protein. In addition, proline is a rigid amino acid due to the covalent binding of its side-chain with the nitrogen main-chain. As a consequence of this unique cyclic side-chain, proline has a significant effect upon the geometry of the backbone chain and also disrupts any regular repeating structure of the three-dimensional conformation of polypeptides. Indeed, proline can act as a structural disruptor for α -helices and as a turning point in β -sheets. As observed in figure 2B, residue Pro222 in the 3β -HSD2 enzyme is located on the edge between an α -helice and a β -sheet. When changed to glutamine or threonine the β -sheet is maintained showing that both variations impose on the protein a drastic conformational change in this region. Both glutamine and threonine have uncharged polar side-chains and are hydrophilic residues, clustering on the outside of proteins. The distinct biochemical properties of proline,

glutamine, and threonine dictate the putative interactions of these amino acids with other surrounding residues, as summarized in figure 2C.

The mutant residues Q222 and T222 suppress hydrophobic interaction with V220, normally observed for P222. As those differences in the structure of residue 222 can affect other regions of the protein, analysis of the substrate-binding and membrane-spanning domains was performed (2,26). No change in the membrane-spanning domain (data not shown) was observed. However, modifications in one substrate-binding region were observed. The 251-274 domain in Q222 and T222 mutant protein presents a β -sheet conformation, while the wild-type has a flexible turn (Figure 3). Considering that 3β -HSD2 enzyme contains only two substrate-binding regions, residues 176-186 and 251-274 (26), this result suggests that reduction in protein activity is mainly due to an impairment of substrate binding. Finally, the native residue P222 appears to be essential for the hydrophobic surfaces on that particular position of the enzyme. Moreover, it seems to establish specific residue interactions that must be critical for the 3β -HSD2 enzyme to achieve the appropriate conformation for its catalytic activity.

In summary, this study has provided further insight concerning the structure-function relationship of 3β -HSD2 mutants. Molecular homology modeling of the mutant 3β -HSD2 showed potential roles for mutated residues. These findings have emphasized codon 222 as an important residue for catalytic activity of the enzyme and allowed us to correlate with biochemical data previously reported (23). Moreover, it can be concluded that structural analysis provides additional insight to the understanding of enzyme action and consequently, it is an important tool for genotype-phenotype correlation.

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