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Fission yeast *Schizosaccharomyces pombe* as a new system for the investigation of corticosterone methyloxidase deficiency-causing mutations

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ABSTRACT

The aldosterone synthase, CYP11B2, catalyses the conversion of 11-deoxycorticosterone to aldosterone, a process that requires three steps: a hydroxylation at position 11β to form corticosterone, another one at position 18 to produce 18-hydroxycorticosterone, and, finally, an oxidation at position 18 to form aldosterone. Aldosterone synthase deficiency usually finds its expression in infancy as a life-threatening electrolyte imbalance, caused by mutations in the *CYP11B2* gene. Therefore, in depth studies of mutations and their enzymatic activities will provide information for the diagnosis and management of hypoaldosteronism caused by CYP11B2 deficiencies. Here, we report the development of a fast and cheap whole-cell technology for the enzymatic characterisation of CYP11B2 mutations. The principle of the new system is the heterologous expression of the mutants of CYP11B2 in fission yeast (*Schizosaccharomyces pombe*) followed by steroid bioconversion assays for the enzymatic characterisation of the investigated mutants.

The new system was validated and 10 known mutations of CYP11B2 have been investigated, two of them for the first time concerning their effect on the CYP11B2 three-step reaction. The results of the fission yeast system were in good agreement with the cell culture results presenting this new system as an alternative non radioactive method that can be applied for the enzymatic characterisation of CYP11B2 mutations.

1. Introduction

Cytochrome P450 enzymes play an important role in steroid hormone biosynthesis of the human adrenal gland, e.g. the production of cortisol and aldosterone. Aldosterone, the most important human mineralocorticoid, is involved in the regulation of the salt and water homeostasis of the body and thus in the regulation of the blood pressure. Aldosterone synthesis in human is mediated through several cytochrome P450 enzymes, among which aldosterone synthase (CYP11B2) catalyses the terminal three steps, the 11β-hydroxylation of 11-deoxycorticosterone (DOC) that leads to corticosterone (B), which is then 18-hydroxylated to yield 18-hydroxycorticosterone (18OHB) and finally oxidized to aldosterone [1,2] (Fig. 1). Deficiencies of aldosterone biosynthesis that are caused by *CYP11B2* gene defects were (and still are) called corticosterone methyl oxidase (CMO) deficiencies (Fig. 1) [3,4]. In CMO I deficiency, the mutations lead to a complete inactivation of CYP11B2 [5,6], or to a significant reduction of its activity [7]. Contrary to CMO I deficiency, the clinical picture of CMO II indicates a blockade of only the terminal 18-oxidation step.

Several studies investigated CYP11B2 in patients suffering from hypoaldosteronism, and series of missense mutations have been reported [8–10], for review see [2,11]. Since hypoaldosteronism patients display usually several missense mutations at the same time [8,10], the investigation of the effect of each single mutation on the activity of CYP11B2 is of high diagnostic and therapeutic interest [8,10,12–14]. However, all reported studies use cell cultures and radioactive substrates for the detection of the steroid bioconversion profile of the investigated CYP11B2 mutants. The estimation of the enzymatic activity of CYP11B2 is carried out either by using radioactive-labelled DOC (or B) and a radioactive detection method [e.g. the high performance thin layer chromatography (HPTLC) and autoradiography] [14,15] or by radioimmunoassay [7,15]. For this reason, neither of these methods can be considered as high or even medium throughput testing system that can be applied to investigate several mutations on the same time. Bureik et al. reported in 2002 the heterologous expression of the human CYP11B2 in fission yeast (*Schizosaccharomyces pombe*) [11]. Surprisingly, CYP11B2 displayed the ability to interact with the endogenous redox partner to support the bioconversion of DOC into B, 18OHB and aldosterone. However, the enzymatic activity of CYP11B2 in the recombinant fission yeast (MB164) was investigated using radioactive-labelled substrates. Therefore, the aim of this study is the development of a new investigation technique that enables the enzymatic characterisation of CYP11B2 mutations on large scale. For this purpose, the

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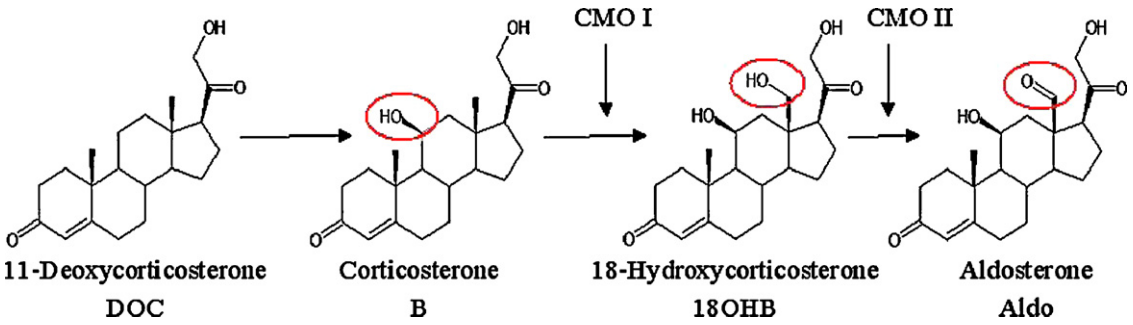

Fig. 1. Reactions catalysed by human CYP11B2. CYP11B2 converts 11-deoxycorticosterone (DOC) via corticosterone (B) and 18-OH-corticosterone (18OHB) to aldosterone (Aldo).

fission yeast system was adapted and coupled with high performance liquid chromatography (HPLC) in order to develop a robust non-radioactive method for the analysis of CYP11B2 mutants.

2. Materials and methods

2.1. Chemicals and enzymes

Steroids were from Sigma (Schnelldorf, Germany). All used organic solvents were of HPLC grade and the water for HPLC analysis was deionised with a Milli-Q Biocel system (Millipore, Bedford). Restriction enzymes were from Promega and NEB.

2.2. Media and general techniques

Media and genetic methods for studying fission yeast have been described in detail [16,17]. Fission yeast cells were cultivated at 30 °C in Edinburgh minimal media (EMM) with supplements as required.

2.3. Molecular biology methods

General DNA methods were performed using standard techniques [18].

The cDNA used in this study as a wild type of *CYP11B2* differs from the sequence reported by Kawamoto et al. [19], at the following positions: Leu106Pro, Arg249Ser, and Glu258Gly. While Arg249Ser is reported as a SNP of *CYP11B2* [20], the variations Leu106Pro and Glu258Gly are changes found in the individual cDNA used here (unpublished results). The expression vector pINT5-*CYP11B2* reported previously for the expression of CYP11B2 in fission yeast [11] was used in this study as a template for the creation of the CYP11B2 mutants and for the expression in fission yeast.

Fission yeast strain MB164 that expresses the wild type of CYP11B2 [11] was used in this work as reference strain to compare the enzymatic activity of CYP11B2 mutants in the fission yeast strains that will be created in this study. For the creation of the CYP11B2 mutants-expressing fission yeast strains, the fission yeast strain NCYC2036 was used like before for MB164 as parental strain.

Mutagenesis of *CYP11B2* was performed using the expression vector pINT5-*CYP11B2* and *Pfu* DNA-Polymerase (Promega; Mannheim, Germany). For the creation of *CYP11B2* missense mutations series of paired oligonucleotides (primers) were designed with the program SILENT SITE SELECTOR (Table 1). The designed primers enable the insertion or deletion of diagnostic restriction endonuclease sites that confirm the creation of the wished mutation in *CYP11B2*. Further confirmation was carried by automatic sequencing (for sequencing primers see Table 1).

2.4. Transformation of fission yeast strain NCYC2036

Transformation of NCYC2036 with CYP11B2 mutant-expressing pINT5 vector was done with cryopreserved fission yeast cells [21]. The pINT5 vector is a shuttle vector containing the pUC-derived sequences (2.0 kb) for use in *Escherichia coli* and the yeast components (6.1 kb) containing the expression cassette, the *ura4* marker, and flanking *leu1* sequences. For the transformation, pINT5-*CYP11B2* was digested with *Not*I to isolate the yeast components containing the CYP11B2 expression cassette, which were subsequently purified by agarose gel electrophoresis. The insertion of the isolated fragment into the yeast was done via homologous recombination of the flanking *leu1* locus fragment into the yeast chromosome. After transformation, the transformed strains with the correctly integrated fragment (in the *leu1* gene in the chromosome) switched its auxotrophic marker from uracil to leucine. They were identified and isolated after replica plating and incubation at 30 °C on EMM plates lacking or containing 0.1 g/l leucine.

2.5. In vivo steroid hydroxylation assays

The bioconversion assay was carried out in 300 ml wide-neck Erlenmeyer flasks covered by a cellulose-pot, where multiple sampling can be done. A fission yeast cell suspension was prepared using fresh EMM with the required supplement of uracil (in the case of the parental strain NCYC2036) or leucine (in the case of the generated strains and the reference strain MB164) to a final concentration of 0.1 g/l. Ten milliliters of cell suspension was transferred to the Erlenmeyer and DOC was added as substrate. The flask was then incubated at 30 °C and 180 rpm, and multiple samples of 500 μl were taken at defined time points and stored at −20 °C until steroid extraction was carried out. The samples were then twice-extracted with an equal volume of chloroform. After evaporation of chloroform the steroids were dissolved in methanol and measured with the HPLC.

2.6. HPLC analysis

HPLC was performed on a Jasco system (Tokyo, Japan) consisting of an auto-sampler AS-2050 plus, pump PU-2080, gradient mixer LG-2080-02 and an UV-detector UV-2075 plus provided with a reversed phase Nucleodur® 100-3C_{18} ec column from Macherey-Nagel. The column temperature was kept constant at 35 °C with a Peltier oven. The mobile phase consisted of MeOH:H_2O 50:50 (A) and methanol (B) and was run at a constant flow of 1 mL/min with a gradient solvent program (Table 2).

Steroids were detected at 240 nm, and peak identification was done using the ChromPass software (V.1.7.403.1, Jasco). Pure steroids (>99%) were used as standards to identify the peaks on HPLC (Fig. 2).

Table 1
Primer sequences used for site-directed mutagenesis and sequencing. Differences in nucleotide sequences between the plasmid pINT5-CYP11B2 and the primers are presented in bold letters. Recognition sites of the enzymes are underlined. The last three lines represent the primers used for sequencing.

Name	Primer sequence	Mutation	Restriction enzyme
K173del_F	5′-GCCAGGGACTTCTCCC AAGCTTGAAGAAGGTGCTGC-3′	K173del: deletion	*Hind*III
K173del_R	5′-GCAGCACCTTCTTCA AAGCTTGGGAGAAGTCCCTGGC-3′	K173del: deletion	*Hind*III
R181Q_F	5′-TGCAGAACGCCCAGGGGAGCCTGAC-3′′	R181Q: cgg → cAg	*Sma*I
R181Q_R	5′-GTCAGGCTCCCCTGGGCGTTCTGCA-3′	R181Q: ccg → cTg	*Sma*I
R181W_F	5′-GCTGCAGAACGCCTGGGGGAGCCTG-3′	R181W: cgg → Tgg	*Sma*I
R181W_R	5′-CAGGCTCCCCCAGGCGTTCTGCAGC-3′	R181W: ccg → ccA	*Sma*I
T185I_F	5′-CCGGGGGAGCCTGATTCTAGA CGTCCAGCCCAG-3′	T185I: acc → aTT	*Xba*I
T185I_R	5′-CTGGGCTGGACGTCTAGAATC AGGCTCCCCCGG-3′	T185I: ggt → AAt	*Xba*I
E198D_F	5′-CATCTTCCACTACACCATAGAT GCTAGCAACTTAGCTCTTTTTGG-3′	E198D: gaa → gaT	*Nhe*I
E198D_R	5′-CAAAAAGAGCTAAGTTGCTAGC ATCTATGGTGTAGTGGAAGATG-3′	E198D: ttc → Atc	*Nhe*I
T318M_F	5′-CTGCAGGGAGCGTCGACATGACAGCGTTTCC-3′	T318M: acg → aTg	*Sal*I
T318M_R	5′-GGAAACGCTGTCATGTCGAC GCTCCCTGCAG-3′	T318M: cgt → cAt	*Sal*I
D335G_F	5′-GGCTCGGAACCCCGGG GTGCAGCAGATCC-3′	D335G: gac → gGG	*Sma*I
D335G_R	5′-GGATCTGCTGCACCCCGGG GTTCCGAGCC-3′	D335G: gtc → CCc	*Sma*I
V386A_F	5′-GTTTTTGGAGCGAGTGGCTAGCTCAGACTTGGTGC-3′	V386A: gtg → gCT	*Nhe*I
V386A_R	5′-GCACCAAGTCTGAGCTAGC CACTCGCTCCAAAAAC-3′	V386A: cac → Agc	*Nhe*I
G435S_F	5′-TAGACATCAGGGGCTCGAGCAGGAACTTCCACC-3′	G435S: ggc → Agc	*Xho*I
G435S_R	5′-GGTGGAAGTTCCTGCTCGAG CCCCTGATGTCTA-3′	G435S: gcc → gcT	*Xho*I
T498A_F	5′-CCTGGCACGTCCCCTCTTCTCGCTTTCAGAGCGAT-3′	T498A: act → Gct	*Ear*I
T498A_R	5′-ATCGCTCTGAAAGCGAGAAGAG GGGACGTGCCAGG-3′	T498A: agt → agC	*Ear*I
hCYP11B2_wt_F+49	5′-GTGGCCAGGGACTTCTCCCAG-3′		
hCYP11B2_wt_F+97	5′-CTGATGACGCTCTTTGAGCTG-3′		
hCYP11B2_wt_R-70	5′-CATGAACATGAGCTGGACGGT-3′		

Table 2
Solvent gradient program for the separation of the steroids converted with CYP11B2.

Time [min]	Phase A [%]	Phase B [%]
0	100	0
15	50	50
18	0	100
20	0	100
25	100	0

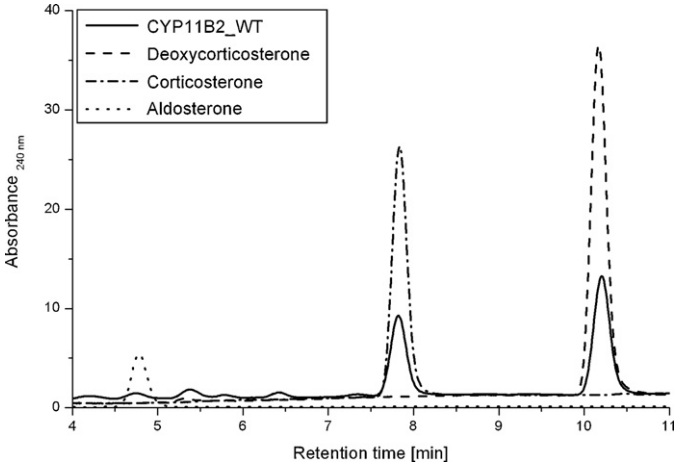

Fig. 2. HPLC chromatograms of the CYP11B2-dependent bioconversion of DOC and separation of pure steroids under the same HPLC conditions.

Since the steroids present in sample are chemically and physically very similar molecules, it was assumed that the relative loss of steroids during the extraction procedure is equal for all steroids. For each steroid we recorded a standard curve. The absorbance of the individual steroids was linearly correlated with the quantity of the steroid measured at 240 nm (data not shown). Therefore, the ratio of products formation can be calculated depending on the intensity signal (I) of steroids of interest and the slope of the respective calibration curve as shown

$$\text{Ratio(products)}[\%] = \frac{I(\text{products})/\text{slope(products)}}{(I(\text{products})/\text{slope(products)} + I(\text{substrate})/\text{slope(substrate)})} \times 100$$

The intensity signal of a steroid is the peak area (A) of a certain steroid in the chromatogram with dimension mV/min. Since DOC is the substrate of the 11β-hydroxylation reaction in which B, 18OHB and aldosterone are produced, the ratio of the 11β-hydroxylation activity of CYP11B2 is calculated as $\sum(\text{B} + 18\text{OHB} + \text{Aldo})/\sum(\text{B} + 18\text{OHB} + \text{Aldo} + \text{DOC})$. Moreover, when B plays the role of the substrate in the second step of the reaction, the 18-hydroxylation, this is calculated as $\sum(18\text{OHB} + \text{Aldo})/\sum(18\text{OHB} + \text{Aldo} + \text{B})$. Finally, when 18OHB is the substrate of the 18-oxidation reaction, this is calculated as $\text{Aldo}/\sum(\text{Aldo} + 18\text{OHB})$. This kind of calculation displays the relative ratios of CYP11B2-mediated product formation and enables the direct comparison of different *S. pombe* strains expressing the different mutants of CYP11B2.

2.7. Statistical analysis

The *t*-test for independent samples was applied to evaluate the differences in the CYP11B2 activity between mutants and wild type. The results of these statistical tests were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Development of fission yeast whole cell system for the investigation of CYP11B2 mutations

The aim of this work was the use of the fission yeast system for the development of a new system that enables the enzymatic characterisation of the CYP11B2 mutants. Several parameters had to be investigated and optimised. For this purpose, the CYP11B2 mutant R181W, which is the first missense mutation of CYP11B2 discovered in hypoaldosteronism patients, was chosen to be expressed in fission yeast and to be enzymatically characterised as a proof of principle (Fig. 3). For the creation of the R181W mutation, a site directed mutagenesis was carried out using the expression vector pINT5-*CYP11B2* and specific primers that delete a diagnostic *Sma*I restriction site (Fig. 3A). After transformation in *E. coli*, the

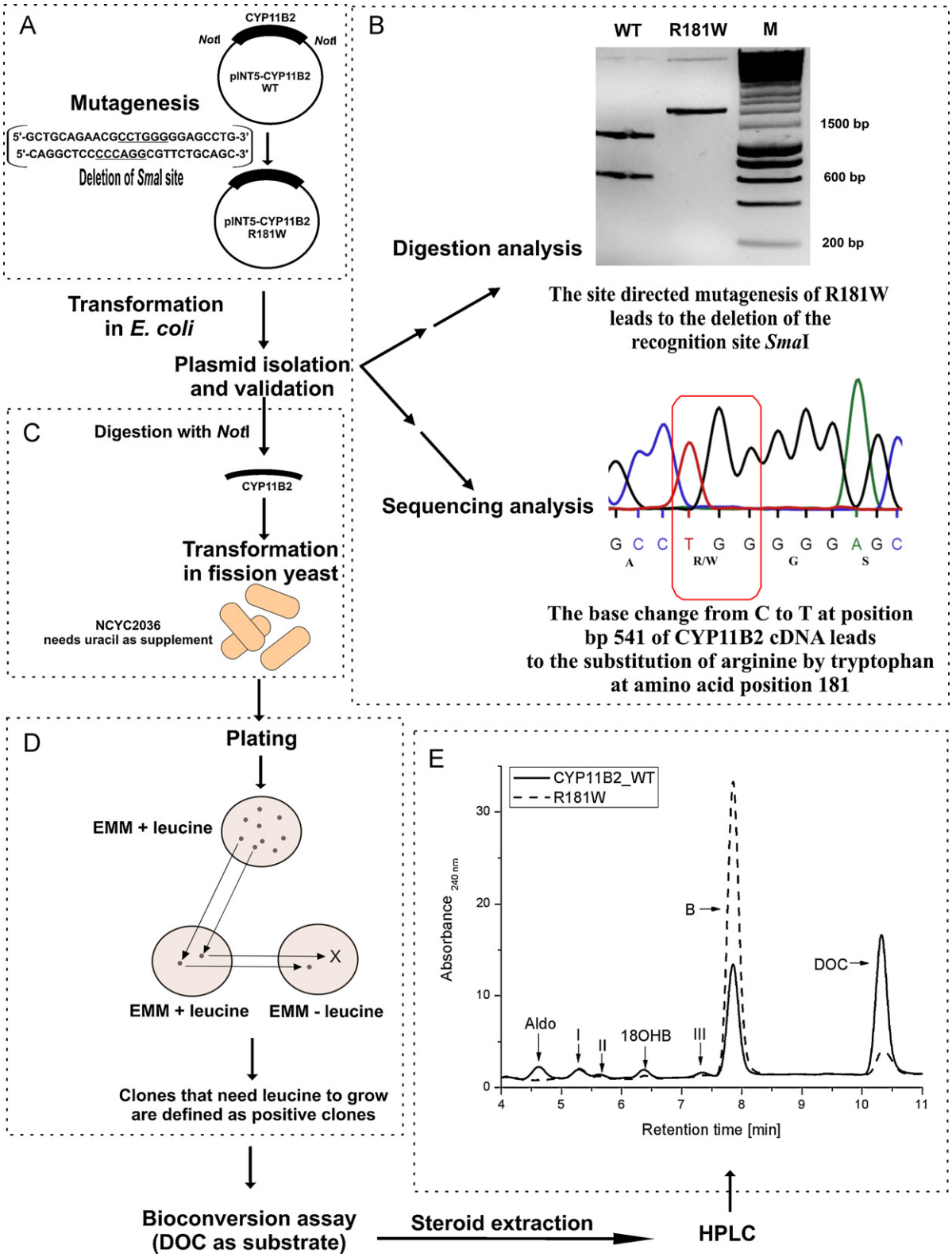

Fig. 3. Description of the fission yeast assay.

plasmid was isolated and the correct change of the codon was verified by digestion analysis with *Sma*I followed by automatic sequencing (Fig. 3B). *CYP11B2* sequence displayed no additional mutations as compared to the wild type sequence mentioned above.

After that, the plasmid was digested with *Not*I and the 6.1 kb fragment containing the CYP11B2 expression cassette flanked with the *leu1* locus of the yeast chromosome was transformed in the fission yeast strain NCYC2036 (Fig. 3C). The transformed cells were plated on EMM plates with leucine. In order to prove if the inserted

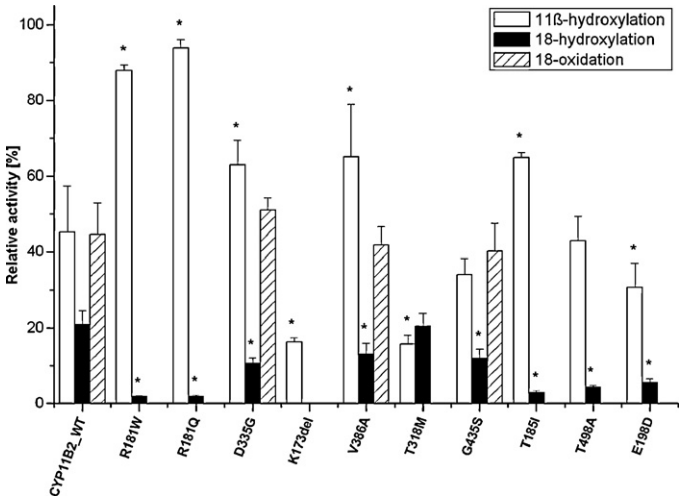

Fig. 4. Relative enzymatic activities of CYP11B2 mutants. 11β-Hydroxylation [$\sum$(B + 18OHB + Aldo)/$\sum$ (B + 18OHB + Aldo + DOC)], 18-hydroxylation [$\sum$(18OHB + Aldo)/$\sum$(18OHB + Aldo + B)] and 18-oxidation [Aldo/$\sum$(Aldo + 18OHB)]. The activities are shown as mean ± SEM ($n \geq 3$). The differences in the CYP11B2 activity between mutants and wild type were evaluated by the *t*-test, significance were marked by *.

fragment was correctly integrated in the leucine gene of the yeast chromosome, multiple replica plating on EMM plates with/without leucine was done and only clones that display growth on leucine-containing medium were defined as positive and picked for further validations (Fig. 3D).

For the enzymatic characterisation of the new CYP11B2 mutant R181W in comparison with the wild type, HPLC was chosen for the separation and measurement of the steroids converted with the CYP11B2 (Fig. 3E).

The parameters of the suggested bioconversion assay were investigated using the fission yeast strain MB164 in order to estimate the cell density of the recombinant fission yeast needed to perform an HPLC-detectable bioconversion of DOC. The investigations showed that a fission yeast suspension with a cell density of 1×10^8 cells/ml displays detectable bioconversion after an incubation time of 24 h with 25 μM DOC as substrate (data not shown).

A bioconversion assay was carried out under the conditions mentioned above in order to compare the enzymatic activity of the CYP11B2 mutation R181W in comparison with the wild type. The HPLC measurement showed clearly that the CYP11B2 R181W mutant expressed in fission yeast interacts with the endogenous redox partner and supports the bioconversion of DOC into B and 18OHB (Fig. 3E). Furthermore, the R181W mutant showed a significantly increased 11β-hydroxylation activity (87.9%) in comparison with the wild type of CYP11B2 (45.2%). However, the subsequent 18-hydroxylation reaction was dramatically decreased (from 20.8% to 1.8%) and no 18-oxidation activity was to be detected (Fig. 4). This result is in good agreement with the results of Pascoe et al., who reported this mutation in hypoaldosteronism patients [8]. Pascoe et al. investigated this missense mutation using transfected COS cells and a radioimmunoassay for the enzymatic quantification of the CYP11B2 activity. As reported here, they found that the R181W mutation displays an increased production of B whereas the 18OHB was decreased and no aldosterone was detected in comparison with the wild type of CYP11B2. This clearly demonstrates that the yeast-based new method is a promising alternative method to cell culture that enables the enzymatic characterisation of CYP11B2 mutants, without the necessity of using radioactive substrates.

3.2. Validation of the new fission yeast system and investigation of CYP11B2 mutations

Since the newly developed yeast-based method for the analysis of CYP11B2 mutants is easy to handle, we performed parallel measurements of, so far, not yet investigated and different already published CYP11B2 CMO II mutants to compare the effects of amino acid replacements under identical experimental conditions. For this, 10 CYP11B2 mutants displaying single mutations were expressed in fission yeast and were characterised following the working schema as summarised in Fig. 3. The enzymatic activities of the CYP11B2 mutants were compared with the enzymatic activity of the wild type of CYP11B2 using fission yeast strain MB164 (Fig. 4).

Recently, we discovered in a CMO II patient a new mutation at the position 181, in which a change from arginine to glutamine (R181Q) has occurred (unpublished results). The enzymatic characterisation of this new mutation using the fission yeast system shows high similarity between the effect of this mutation and the R181W mutation. Mutant R181Q displayed an increased 11β-hydroxylation (93.8%) and decreased 18-hydroxylation activity (1.8%) and no 18-oxidation activity, similar to R181W. This observation shows the importance of the position 181 for the enzymatic activity of CYP11B2.

The mutant D335G was previously reported in a heterozygous patient in association with CMO II and it has not yet been characterised [22]. Therefore, the enzymatic characterisation of this mutation using the fission yeast system will enable us to understand the effect of this point mutation on the activity of CYP11B2. As shown in Fig. 4, this mutant displayed an increase of the 11β-hydroxylation activity (63.0%) whereas the 18-hydroxylation activity was strongly decreased (10.6%). Interestingly, the 18-oxidation activity was not affected by the mutation and displayed almost similar 18-oxidation activity in comparison with the wild type of CYP11B2.

In addition to the missense mutations mentioned above, a deletion mutant of CYP11B2 was also characterised using this fission yeast system. Mutation R173del has been reported in CMO II patients [9], but the effect of this deletion has also not been studied so far. Interestingly, the amino acid arginine at position 173 is often replaced with lysine and published to be a single nucleotide polymorphism (SNP) [19]. For this reason and since the wild type of CYP11B2 used in this study has this polymorphism, the lysine 173 has been deleted and the resultant mutation K173del has been characterised. The deletion mutation K173del displayed in the fission yeast a strongly decreased 11β-hydroxylation activity (16.3%). Furthermore, this deletion mutant displayed no 18-hydroxylation and 18-oxidation activities.

For further validation of the new investigation system, already characterised CYP11B2 mutants have been re-investigated using the fission yeast.

The V386A mutation, which is usually combined with the R181W mutation in CMO II deficiency patients, displayed in this study a slight increase in the 11β-hydroxylation activity (65.1%) whereas the 18-hydroxylation was decreased (13.0%) without any change in the 18-oxidation activity. These findings agree with the results of Pascoe et al. who reported the combined inherence of this mutation with the R181W mutation in CMO II deficiency patients and that the clinical phenotype required the homozygous presence of both mutations [8]. The findings of Pascoe et al. showed that V386A itself caused a small but steady reduction of the 18-hydroxylation activity while the 11β-hydroxylation and the 18-oxidation activities were similar to that of the wild type. Furthermore, several studies use this double mutation R181W/V386A as positive control for the investigation of CMO II deficiency patients [10,15].

Mutant T318M showed a strong decrease in the 11β-hydroxylation activity (15.7%), while the 18-hydroxylation activity was not affected and the 18-oxidation activity was completely abolished. Zhang et al. investigated this mutation using MA-10 cells. However, the 11β-hydroxylation and 18-hydroxylation were not determined and only the amount of aldosterone was estimated by immunoassay. As a result, they reported that T318M displayed lower 18-oxidase activity in comparison with the reference mutant R181W/V386A [10] and they concluded that T318M alone is sufficient to eliminate the aldosterone synthase activity. This previous observation agrees with our results using the fission yeast system.

In contrast to the results of Kuribayashi et al. [23], who investigated the mutation G435S and found that this mutation displays no 18-oxidation activity, this mutation displayed a decreased 18-hydroxylation activity (11.9%) in the fission yeast system whereas the 11β-hydroxylation and 18-oxidation activities were unaffected in comparison with the wild type of CYP11B2. The absence of aldosterone in the case of the mutation G435S in the study of Kuribayashi can be possibly due to the very short time of incubation (20 min) with the substrate DOC using mitochondrial proteins isolated from COS-7 cells.

The mutations T185I and T498A showed similar 18-hydroxylation/oxidation profiles after investigation in the fission yeast system compared with the cell culture. In contrast to this, the 11β-hydroxylation activity in mutant T185I seemed to be increased (64.9% compared with 45.2% for the wild type) whereas mutant T498A displayed no significant change in the 11β-hydroxylation activity compared to the wild type (42.9%). Both mutants displayed decreased 18-hydroxylation activity (T185I: 2.9%, T498A: 4.3%) and no 18-oxidation activity. Dunlop et al. investigated these mutations using transfected COS-1 cells and thin layer chromatography (TLC) as well as radioimmunoassay. The transfected COS-1 cells were able to produce corticosterone and 18-hydroxycorticosterone, but hardly aldosterone [15].

Finally, the missense mutation E198D has been reported to reduce the CYP11B2 activity, and to abolish the 18-oxidase activity with residual 11β-hydroxylation and 18-hydroxylation activities in the combination with other single mutations of CYP11B2 [7]. E198D in the fission yeast system showed that the mutation displays decreased 11β-hydroxylation (30.7%) and 18-hydroxylation (5.5%) activities, which are in good agreement with the reported results [7]. Our results differ from the previous observation in that we could not detect aldosterone by HPLC, whereas those authors found some residual activity although it was significantly decreased and had not been quantified.

Taken together, a new investigation system for the enzymatic characterisation of CYP11B2 has been developed (Fig. 3). The new system has been validated and several CYP11B2 mutations have been investigated for the first time using this system. Moreover, the results of the enzymatic characterisation of these mutations in the fission yeast system are in good agreement with cell culture results. Since the investigated mutations are positioned in different regions of the CYP11B2, the new investigation system is convenient for the investigation of all CMO II (and other) mutations regardless of their positions in the CYP11B2 protein.

Taking into account the advantages and disadvantages of this new system in comparison with the previously used cell culture systems, it is clear that the new system is a non-radioactive method with low cost in comparison with cell culture. Moreover, and since transient transfection is usually preferred and carried out to create recombinant cell lines, the creation of stable transformed recombinant fission yeast strains has the advantage that this recombinant microorganism can be frozen and reused many times in contrast to the transient transfected cell lines. This way, the effect of any new mutation on the activity of CYP11B2 can be easily compared with the effect of other mutations already characterised. Furthermore, we could show recently the ability to use recombinant fission yeast for the development of medium throughput screening systems using the 96-well plate format [24]. Therefore, it is possible to optimise the new investigation method reported in this study to be applied in 96-well plate in order to investigate CYP11B2 mutations on large scale.

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