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'Chemical co-substrate rescue' of phytanoyl-CoA 2-hydroxylase mutants causing Refsum's Disease

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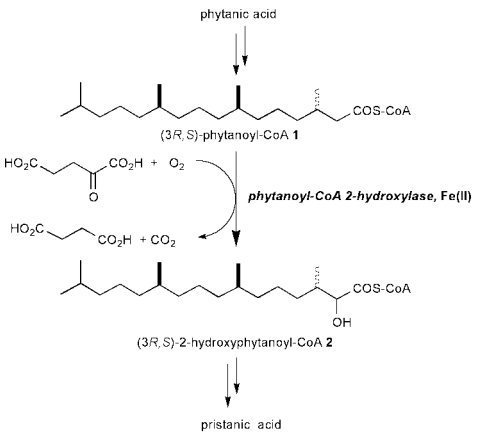
The *in vitro* catalytic activity of two clinically observed mutants of phytanoyl-CoA 2-hydroxylase, an iron(II)/2-oxoglutarate-dependent oxygenase causing Refsum's Disease, was partially rescued by the use of alternatives to the natural cosubstrate, 2-oxoglutarate; this is the first demonstration of 'chemical co-substrate rescue' of mutations to an enzyme causing human disease.

Phytanic acid in the human diet is derived from the phytol sidechain of chlorophyll, but the presence of a 3-methyl group prevents its degradation *via* the fatty acid β-oxidation pathway. Instead, a preliminary pathway effects α-oxidation of phytanic acid, excising a methylene group to give pristanic acid.[1,2] Within this pathway, hydroxylation of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA is catalysed by phytanoyl-CoA 2-hydroxylase (PAHX),[3,4] an iron(II) and 2-oxoglutarate-dependent oxygenase (Scheme 1).[5] Mutations to PAHX cause *ca.* 45% of adult Refsum's Disease (ARD) with other cases being associated with a second locus.[6]

Two common point mutations in human PAHX, R275W and R275Q, are present at an allele frequency of 3/40 (7.5%) and 5/40 (12.5%), respectively, in Dutch/Scandanavian patients with ARD.[3,7] Sequence analyses revealed that Arg-275 is conserved in all reported PAHX enzymes and closely related sequences. Analysis of the sequences in the light of crystal structures[8,9] for two other 2-oxoglutarate oxygenases suggested that Arg-275 binds the 5-carboxylate of the co-substrate *via* an electrostatic interaction.[8] We postulated that the PAHX R275Q and R275W mutants were inactive due to defective 2-oxoglutarate binding or utilisation, and that it may be possible to rescue their activity using alternative co-substrates.

The two clinically observed mutants of Arg-275 in human PAHX (R275Q and R275W)[3,7] and a further mutant R275A, made for comparison, were constructed by site-directed mutagenesis. The desired proteins were expressed *via* standard techniques in recombinant *E. coli* and purified as mature (*i.e.* without their peroxisomal targeting sequences) enzymes to > 95% homogeneity (by SDS-PAGE analysis). Mutations were confirmed by DNA sequencing and ESI MS analyses. Analysis of the secondary structure by circular dichroism suggested that all mutants had a similar overall structure to the wild-type enzyme.



Scheme 1 The role of phytanoyl-CoA 2-hydroxylase in the peroxisomal degradation of phytanic acid.

Activity of the wild-type enzyme and mutants was assayed by conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA.[10] Assays were performed (at least in triplicate) according to a modified version of a published procedure[11] and included ATP to obtain maximum activity.[12] The activity was further enhanced *ca.* two-fold by the use of tris(carboxyethyl)phosphine (TCEP) in place of the previously used dithiothreitol (DTT).[11] Two concentrations of 2-oxoacid (2 and 60 mM) were used in order to facilitate detection of low levels of activity. Assay mixtures for the analogues contained: 50 mM Tris-HCl, pH 7.5, 1 mM $FeSO_4$, 50 μM synthetic (3*RS*,7*R*,11*R*)-phytanoyl-CoA, 0.44 mM β-cyclodextrin, 100 μM TCEP, 10 mM ascorbate, 4 mM ATP and *ca.* 20 μg enzyme (2 mM 2-oxoacid) or 10 μg enzyme (60 mM 2-oxoacid). Reactions were quenched with 250 mM EDTA after incubation for 60 min (2 mM 2-oxoacid) or 5 min (60 mM 2-oxoacid). Samples were centrifuged and analysed by HPLC using a Hypersil C_{18} column (250 × 4.6 mm) monitoring at 254 nm.[11]

Using the natural co-substrate, 2-oxoglutarate, the activity of the two clinically observed mutants was < 0.5% of that of the wild-type enzyme (Table 1). A range of 2-oxoacids was tested in an attempt to restore the activity of the mutants. Homogentisate, 4-hydroxyphenylpyruvate, indole-3-pyruvate, 2-mercaptosuccinate all gave specific activities of < 0.2 nmol min^{-1} mg^{-1} with wild-type enzyme and all mutants. The activity of the R275Q and R275W mutants was significantly 'rescued' compared to the wild-type activity with 2-oxoglutarate as a co-substrate using certain 2-oxoacids at a concentration of 60 mM (Table 1). Optimum rescue of mutants with hydrophobic/aliphatic residues in place of Arg-275 is achieved using 2-oxoacids with side-chains of 2–4 carbon atoms or equivalent length side-chains. Effective examples are in the use of 2-oxobutyrate with R275Q mutant (Table 1, entry 4) and 2-oxo-5-thiahexanoate with the R275W mutant (Table 1, entry 9). The latter case is striking because 2-oxo-5-thiahexanoate cannot be substituted for 2-oxoglutarate (< 0.5%) in assays with the wild-type enzyme. The dramatic change in co-substrate selectivity resulting from PAHX Arg-275 mutations may be useful for the clinical identification of these particular mutants using a modified assay with alternative 2-oxoacids.

Even higher levels of rescue were obtained in the case of the R275A mutant where most of the hydrophobic analogues tested (Table 1, entries 3–9), led to 22–28% rescue at 60 mM. An exception was 2-oxooctanoic acid (Table 1, entry 10), which was inactive with the wild-type enzyme, and all mutants assayed, presumably due to its carbon chain being too large to

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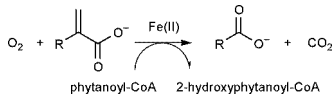
Table 1 Specific activities of (3*RS*,7*R*,11*R*)-phytanoyl-CoA hydroxylation as catalysed by mature recombinant wild-type and mutant PAHX enzymes in the presence of various 2-oxoacids (nmol min^{-1} mg^{-1} protein). Assays were carried out with 2 mM or 60 mM 2-oxoacid

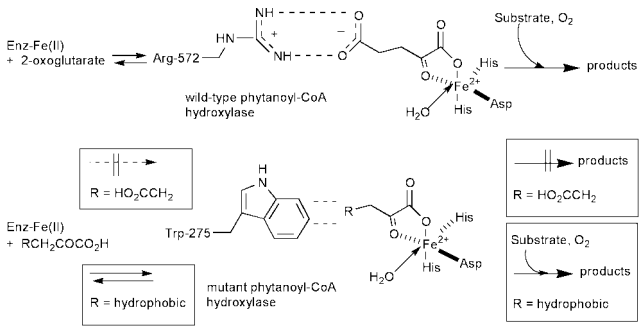


PAHX mutant	R275 (wild-type)		R275A		R275Q		R275W	
2-oxoacid/mM	2	60	2	60	2	60	2	60
1. 2-oxoglutarate R = $(CH_2)_2CO_2^-$	265	885	2.63	128	0.59	< 0.2	< 0.2	< 0.2
2. 2-oxoadipate R = $(CH_2)_3CO_2^-$	129	235	3.14	57.0	< 0.2	< 0.2	< 0.2	< 0.2
3. pyruvate R = CH_3	6.8	78.6	8.7	201	< 0.2	54.0	0.6	11.0
4. 2-oxobutyrate R = CH_2CH_3	2.1	69.4	5.7	250	2.7	106	0.9	55.2
5. 2-oxovalerate R = $(CH_2)_2CH_3$	1.6	40.1	4.8	210	3.0	71.6	1.93	63.9
6. 2-oxoisovalerate R = $CH(CH_3)_2$	0.65	45.3	4.30	192	1.52	72.2	0.49	17.0
7. 2-oxocaproate R = $(CH_2)_3CH_3$	0.7	20.1	5.3	235	1.33	42.4	2.4	66.2
8. 2-oxoisocaproate R = $CH_2CH(CH_3)_2$	0.8	17.8	4.8	217	1.9	45.4	2.2	56.2
9. 2-oxo-5-thiahexanoate R = $(CH_2)_2SCH_3$	< 0.2	< 0.2	4.8	226	1.6	52.8	2.6	98.2
10. 2-oxooctanoic acid R = $(CH_2)_5CH_3$	N/D	< 0.2	N/D	< 0.2	N/D	< 0.2	N/D	< 0.2

be accommodated in the proper orientation in the active site. The R275A mutant was also less selective than the wild-type or other tested mutants, presumably due to a relaxation of steric and electrostatic constraints reflecting the presence of a small, hydrophobic and neutral side-chain.

2-Oxoacids are metabolically related to proteinogenic amino acids *via* transamination reactions. Several of the 2-oxoacids which rescue the activity of the clinically observed R275W and R275Q mutants are thus accessible *via in vivo* amino acids, *e.g.* 2-oxovalerate from valine and 2-oxo-5-thiahexanoate from methionine. Thus, certain forms of ARD might be treated *via* dietary supplements containing the appropriate amino acids. Maple syrup urine disease is caused by a deleterious accumulation of excess 2-oxacids, and is treated, in the reverse of this proposed therapy, by a diet low in branched-chain amino acids.[13] Restoration of complete wild-type activity may not be required, as 5% of wild-type activity is apparently sufficient to effectively correct inherited homocystinuria[14] with vitamin B6.

To our knowledge the only other example of the rescue of enzyme activity with a modified co-substrate has involved the elegant use of ATP analogues to study *in vivo* activity of



Scheme 2 'Chemical co-substrate rescue' of a PAHX mutant as exemplified for R275W. R = hydrophobic/aliphatic group. Wild-type enzyme showing interaction of guanidino group of Arg-275 and 5-carboxylate of 2-oxoglutarate (above). Unfavourable interaction between aromatic sidechain of Trp-275 and 5-carboxylate of 2-oxoglutarate; rescue of activity *via* hydrophobic interactions in 2-oxoacid binding site (below). The relative arrangement of the iron ligands is that of deacetoxycephalosporin C synthase (DAOCS).[8]

kinases.[15] The *in vitro* work that is reported here is the first demonstration of the 'chemical co-substrate rescue' of mutations in an enzyme (Scheme 2) implicated in a human disease. *In vivo* studies directed towards demonstrating the technique in cell lines are in progress.

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