Mutants of Phenylalanine Dehydrogenase: New Bio-Catalysts in the Asymmetric Synthesis and Racemic Resolution of Non-Natural Amino Acids

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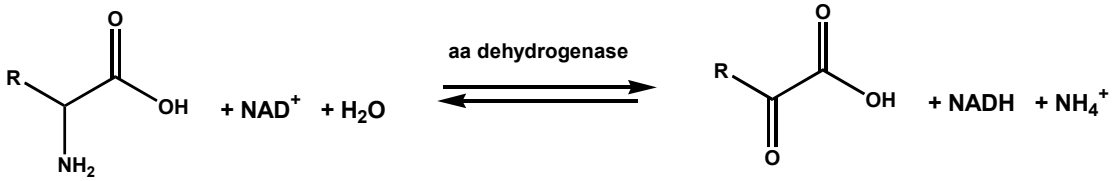
The annual world market for chiral synthons was estimated in 2003 to be well in excess of $10[11]. Among such compounds, non-natural amino acids are important as building blocks for synthesis of alkaloids, peptides and other pharmacological agents e.g. ACE and HIV-protease inhibitors. Whereas the L-enantiomers of naturally–occurring amino acids are cheaply available through biological processes, non-natural amino acids must be made either through a process of asymmetric synthesis or by resolving the components of a racemic DL-mixture. Chemical asymmetric synthesis, however, usually involves heavy-metal catalysts with attendant cost and pollution issues, and the percentage enantiomeric excess rarely meets ideal requirements. Enzymes, by contrast, are clean, operate under mild conditions, and usually deliver 100% ee. A variety of enzyme-catalysed reactions have indeed been used in the production of optically pure amino acids.

Barriers to more widespread use of these seemingly ideal catalysts have been i) cost of production, ii) fragility, and above all iii) limited and sometimes inappropriate specificity. The first problem has been solved in recent years by gene cloning and highly efficient methods of ‘over-production’ of enzyme proteins. The second has in many cases been overcome by searching for suitable biological source organisms, especially among extremophiles, which by definition must produce enzymes able to withstand the same extremes of temperature, pH, salinity etc. as the whole organism. The most challenging issue is that of specificity since the range, sometimes very narrow, of substrates that the enzyme has evolved to handle in its biological context may not closely match those desired by the synthetic chemist, even if the type of reaction is highly desirable – e.g. aldol condensation.



Our work has been directed at overcoming this third barrier, the barrier of limited specificity, in the case of the amino acid dehydrogenases. This group of enzymes interconverts L-amino acids and 2-oxoacids. The reaction is a reversible oxidative deamination, releasing ammonia and using the coenzyme NAD^+ as the oxidant. In the reverse direction, therefore, a new chiral centre is created at the α-carbon atom. Amino acid dehydrogenases fall into two families, the glycine and alanine dehydrogenases forming one group and a much larger set, with no sequence similarity to the first, forming the second. The archetype of the second group is glutamate dehydrogenase; because of its metabolic importance it is very widely distributed in Nature and has been extensively studied; also it was the first member of the family to have its 3D structure solved at high resolution by X-ray crystallography [1,2]. Recognition of the family relationship and sequence similarity to phenylalanine (PheDH), leucine (LeuDH), valine and lysine dehydrogenases led to the suggestion that molecular modeling might provide an adequate basis for protein engineering of these other enzymes [3]. This has proved to be strikingly successful, with two targeted point mutations, G124A and L307V, shifting the specificity of PheDH towards that of LeuDH [4,5], and another single mutation, replacing N145 by any one of A,I,L or V, introducing a 50-fold discrimination between phenylalanine and its 4-OH analogue tyrosine as substrates [6]. The 3D structure for a PheDH has meanwhile been solved [7], but it is important for the future of protein engineering in this area to know that homology modelling can be the basis of an excellent result. This should give confidence in using amino acid dehydrogenase starting points for which a directly-solved structure is not yet available.

We were also anxious to discover how far these and other mutations might open up the active site to interesting non-natural amino/oxo acid substrates [8]. In our starting form of PheDH, the wild-type enzyme from *Bacillus sphaericus*, the polar asparagine residue at position 145 is relatively unfavourable for binding of non-polar substrates. We have found that its replacement by non-polar alternatives in the set of mutants mentioned above not only favours Phe as a substrate but creates efficient catalysts also for a range of non-natural substrates that are only poorly handled by the wild-type enzyme [9]. For example, activity of the wild-type PheDH with 4-trifluoromethyl phenylpyruvate is only 1.8 U/mg compared to 29U/mg with the N145A mutant. This study also showed, however, that even the wild-type enzyme shows remarkably good activity with a range of non-natural substrates that had not previously been tested – pyridyl pyruvate (18U/mg), 3-cyclohexyl pyruvate (12U/mg), 4-fluorophenylpyruvate (228 U/mg – higher than the rate with the physiological substrate) etc.

These studies have established, therefore that this enzyme and its various derived mutants provide a versatile set of biocatalysts able to handle a wide range of oxo/amino acid substrates. Their effective use requires several issues to be addressed:-

1. Can the enzyme be efficiently produced in host cells?

2. Can the host cells be grown to high cell density while retaining good enzyme expression?
3. Can the enzyme be easily purified and stabilized or else used in a partially purified form?
4. Can the enzyme be immobilized, allowing multiple use?
5. Can the cofactor NAD(H) be recycled in an economic way that is compatible with the requirements of the primary reaction?
6. Can the necessary substrate for the reaction be readily accessed or synthesized?
7. In the case of poorly water-soluble substrates will the enzyme tolerate organic solvents?
8. Is enantioselectivity in any way compromised in using these modified biocatalysts?

Subcloning the PheDH of *Bacillus sphaericus* into the expression vector ptac 85 [4] gave a construct allowing reproducible expression of the enzyme and its engineered mutants at high levels. On a laboratory scale a 1 litre culture of *E. coli* readily yielded upwards of 30 mg pure enzyme following dye-ligand and hydrophobic chromatography. Elsewhere in this meeting [10,11] we describe the engineering of growth conditions in fed-batch mode to provide at least a 40-fold increase in biomass yield without any loss in enzyme yield per gm cells. The enzyme as purified shows good long-term stability properties, but, in view of the high level of expression, it is also possible to use whole cell preparations e.g. by bead entrapment.

A central issue is whether to use the catalyst to introduce the chiral centre into an oxoacid prepared by chemical synthesis or whether instead to start with a racemic amino acid mixture. In the latter case, the enzyme catalyst can be used to selectively and quantitatively oxidise the L-amino acid, leaving the D-amino acid behind to be separated from the reaction mixture. The oxoacid that is produced in this reaction can likewise be separated and, in a second phase of catalysis be reductively aminated to the enantiomerically pure L-amino acid. The choice will depend on substrate availability. We have described elsewhere successful routes to synthesis of the oxoacid substrates [9], but there may be cases where the racemic amino acid is more readily accessible and this application would have the merit of delivering both enantiomers.

Both these applications of the reaction in effect depend on the ability to recycle the nucleotide cofactor as this is too costly an item to use stoichiometrically and discard. In the direction of reductive amination we have found that yeast alcohol dehydrogenase and 5% EtOH provides an effective way of recycling NAD^+ that can be used in the same reaction mixture as the amino acid dehydrogenase reaction and drives the latter to completion at pH 8.5. The ethanol serves a dual function, in that it also improves the solubility of some oxoacid substrates. In the reverse reaction the requirement is for reoxidation of NADH. We run these reactions at pH 9.5 and have achieved efficient recycling by

using a bacterial diaphorase, which requires only air-derived oxygen as the terminal acceptor, and a dye coupling agent.

A significant potential problem is substrate solubility. As mentioned, 5% ethanol is tolerated, but a very promising observation [12] is the stability of the catalyst in two-phase applications, where most of the substrate is in an immiscible non-polar phase, such as hexane, and this upper layer serves as a reservoir, feeding substrate to the aqueous layer containing enzyme and cofactor, as fast as it is used up. This should open up the application of these biocatalysts to a wide range of substrates.

Finally, all our reactions have been monitored by chiral HPLC and thus far there has been no indication of any departure from 100% enantioselectivity for the L-isomer.

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