9 Dehydrogenases – Characteristics, Design of Reaction Conditions, and Applications

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1 Introduction

There is an increasing trend in the pharmaceutical and agrochemical industries, to develop products containing enantiomerically pure materials. This trend was accelerated by a decision of the American Food and Drug Administration (FDA) in May 1992. Safety information is now demanded for individual stereoisomers of products submitted for approval and although racemates will still be continued to be approved on a case by case basis, information on each of the enantiomers is required. This significantly increases the cost of generating the necessary data for approval of racemates. This regulatory environment has led to an increase in the number of single isomer drugs being approved. In the current world market for pharmaceuticals, it is estimated that around 20% ($50 billion) is accounted for by optically pure drugs. This percentage is expected to increase to over 30% by the year 2000.

As a result of the growth in demand for chiral actives, the market for chiral intermediates is currently estimated in the region of $1 billion per annum worldwide, and growing fast. This has provided an enormous impetus for the development of enantioselective chemical transformations, such as the reduction of carbonyl compounds.

A range of reagents for the asymmetric reduction of carbonyl compounds is available. They can be divided into two groups: stoichiometric and catalytic reagents (for reviews see: SINGH, 1992; HARADA and MUNEGUMI, 1991; NISHIZAWA and NOYORI, 1991). Although in many cases high enantioselectivity can be achieved, these reagents still have limitations; Nonactivated carbonyl compounds may not be reduced or only slowly with low enantioselectivity (e.g., alpine borane (1)), they can show a limited substrate tolerance (e.g., chlorodiospinocampheylborane (2), the synthesis of some of these agents is laborious (e.g., R,R-2,5-dimethylyborolane (3); MASAMUNE et al., 1986), they have to be used at low temperatures (−78°C to −100°C, BINAL-H) or at high pressure (e.g., 100 bar hydrogen, BINAP-Ru (4); NOYORI, 1989, 1990). The oxazaborolidine (5) reduces aryl alkyl ketones with high enantioselectivity, but gives poorer results with other ketones (DOUGLAS et al., 1996).

Although a number of chemical agents for the reduction of carbonyl compounds is available (Fig. 1), from both the academic and industrial viewpoints, it is likely that biotransformations will have an important role in the preparation of chiral intermediates over the medium term. There is considerable interest in the application of enzymes or whole cells for the reduction of carbonyl compounds, since they act under mild conditions and show high regio- and stereoselectivity (HOSONO et al., 1990).

The first report of the use of yeast cells as a catalyst for asymmetric reductions was the reduction of a carbonyl compound by fermenting bakers’ yeast in 1918 by NEUBERG and LEWITE (see Chapter 8). A broad range of bacteria, yeasts, and fungi has been used for the asymmetric reduction of carbyonyls (CHRISTEN et al., 1992; AZERAD and BUSSON, 1992; BESSE et al., 1994; GUNTHER and SIMON, 1985; WARRHURST and FEWSON, 1994; YAMADA and SHIMIZU, 1988).

Today, about 3500 enzymes are known (Enzyme Nomenclature 1992, Academic Press), of which about 70% are cofactor-dependent and about 15% are commercially available. The above mentioned microbial reductions are performed by redox enzymes (EC 1). The substrate that is oxidized is regarded as hydrogen donor. The recommended name is dehydrogenase or reductase. The term oxidase is only used in cases where O2 is the acceptor, and peroxidases use H2O2 as acceptor. More than 650 oxidoreductases are known (DAVIES et al., 1989) of which about 90 are commercially available. About 80% of all redox enzymes use nicotinamide adenine dinucleotide (NAD 6a, NADH 7a) as coenzyme redox “partners” and a further 10% the corresponding phosphates (NADP 6b, NADPH 7b) (Fig. 2). In most organisms, NAD is employed in oxidative catabolism linked to ATP production whereas NADPH is utilized for reductions involved in the biosynthesis (anabolism) of cell structures or energy storage (e.g., steroids or fatty acids).

The abbreviations NAD(H) and NADP(H) are used to simultaneously refer to the oxidized and reduced forms of the same coenzyme (i.e., 6a/7a, 6b/7b), whereas NAD(P) and NAD(P)H refer to the oxidized and reduced
forms respectively of both coenzymes (i.e., 6a/6b; 7a/7b). NAD(P)(H) refers to all four co-
enzymes. NAD (6a) is a pyridinium salt and hence is frequently denoted as NAD\(^+\), whereas NADH is neutral according to this specification. However, except under the most acidic conditions the pyrophosphate bridge bears two negative charges and the phosphate group of NADP(H) will also be deprotonated, hence overall the molecule has negative charge.

Recent literature tends not to show the charges with the abbreviations and this convention will be adhered to in this chapter unless the presence of the charge is essential to the argument. This has the advantage that it avoids ambiguities inherent in the consensus abbreviations, e.g., NAD(H), NAD(P)(H).

The classification of NAD(P)-dependent oxidoreductases is shown in Tab. 1. This group is also the most important for preparative applications (WANG and KING, 1979). Flavins (FMN, FAD) and pyrroloquinoline quinone (PQQ) are involved more rarely. Oxidoreductions cover about 20–25% of the publications and patents (SEEBACH, 1990; FABER, 1995). The Chapman & Hall Biotransformation Database (1996) contains about 1040 reactions catalyzed by dehydrogenases. The number of reactions published on dehydrogenases over the past 20 years is summarized in Fig. 3.

Typical dehydrogenase reactions are depicted in Fig. 4. It is not intended to give an exhaustive review of all redox reactions catalyzed by alcohol dehydrogenases. These are much easier accessible in reaction databases on biotransformation (e.g., Chapman & Hall, SynopSys). Rather, this chapter is written from the viewpoint of a synthetic chemist, who wants to reduce a given carbonyl compound by means of a biotransformation. Several questions will be addressed consecutively in the following sections:

- What are the advantages of isolated enzymes versus whole cells?
- What are best systems for the regeneration of expensive coenzymes?
- What are the alternative routes to the direct reduction of a carbonyl compound and how are they assessed?
- Can oxidoreductases be used in organic solvents? What alternatives are there?

Fig. 1. Some reagents used in enantioselective chemical reductions of carbonyl compounds.
1 Introduction

– Which types of reactors can be used for continuous enzymatic synthesis?
– Is the stereochemical outcome of an enzymatic reduction predictable? What are the kinetic limitations and how can they be circumvented?
– Which commercial and noncommercial dehydrogenases are available? What are their characteristics? Which of them are most recommended for a primary screening?
– Which dehydrogenases have already been applied on a large scale?

This chapter will focus on the isolated alcohol dehydrogenases whereas microbial transformations and oxygenases will be addressed in the preceding and the following chapters, respectively. Parts of the topics addressed here have been reviewed in Vol. 6a of the First Edition of Biotechnology (Rehm and Reed, 1984) and by Jones (1986), Yamada and Shimizu

Fig. 2. Nicotinamide adenine dinucleotide coenzymes.

Tab. 1. Classification of NAD(P)-Dependent Oxidoreductases According to Enzyme Nomenclature

<table>
<thead>
<tr>
<th>Number</th>
<th>Donor</th>
<th>Number of Enzyme Subclasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.1</td>
<td>−CH−OH</td>
<td>208</td>
</tr>
<tr>
<td>1.2.1</td>
<td>CHO, C=C=O</td>
<td>48</td>
</tr>
<tr>
<td>1.3.1</td>
<td>−CH−CH−</td>
<td>35</td>
</tr>
<tr>
<td>1.4.1</td>
<td>−CH−NH₂</td>
<td>17</td>
</tr>
<tr>
<td>1.5.1</td>
<td>−CH−NH−</td>
<td>21</td>
</tr>
<tr>
<td>1.6.1</td>
<td>NAD⁺</td>
<td>1</td>
</tr>
<tr>
<td>1.8.1</td>
<td>sulfur</td>
<td>4</td>
</tr>
<tr>
<td>1.10.1</td>
<td>diphenols</td>
<td>1</td>
</tr>
<tr>
<td>1.12.1</td>
<td>hydrogen</td>
<td>1</td>
</tr>
<tr>
<td>1.11.1</td>
<td>peroxidases</td>
<td>11</td>
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<tr>
<td>1.13.11</td>
<td>dioxygenases</td>
<td>36</td>
</tr>
<tr>
<td>1.13.12</td>
<td>mono-oxygenases</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 3. Number of publications on reactions catalyzed by dehydrogenases.
2 Advantages and Disadvantages of Whole-Cell and Enzymatic Transformations

In the course of the first systematic investigations on the stereochemistry of bakers’ yeast reductions of carbonyl compounds, MACLEOD and coworkers (1964) found that (S)-alcohols were formed with moderate selectivities. Subsequently, many papers were published addressing asymmetric reductions catalyzed by bakers’ yeast (cf. Chapter 8, this volume). Bakers’ yeast shows a very broad substrate spectrum and in many cases good or high enantio- and diastereoselectivity, however, problems are often encountered (Fig. 5).

Biological systems are genetically variable. The enzymes involved in redox reactions are not necessarily the same in different strains of the same organism. In order to reproduce published results it is essential to employ exactly the same strain (CHRISTEN and CROUT, 1988; CHEN et al., 1984). For example, in the reduction of 4-chloro-3-oxobutanoic acid methyl ester catalyzed by bakers’ yeast from Oriental Yeast Co. (NAKAMURA et al., 1985) an (R)-se-
lectivity was obtained, whereas the same reduction catalyzed by bakers’ yeast from Red Star Co. yielded the (S)-enantiomer (ZHOU et al., 1983).

Moreover, the cultivation conditions (USHIO et al., 1986), the age, and the metabolic status of the organisms have to be the same to reproduce published results because of physiological variabilities (EHRLER et al., 1986). The expression of enzymes, which are responsible for a desired biotransformation, depends on the cultivation conditions of the organisms. For economical reasons, the cell produces some enzymes only, if an essential nutrient is not present in the medium. Some enzymes are controlled by “catabolite repression”, which means that in the presence of glucose their formation is repressed, whereas during growth on another carbon source, these enzymes are expressed. Last but not least, the final product of a biochemical pathway can also affect the expression of enzymes involved in this pathway (end product repression).

Transport of carbonyl or hydroxy compounds into and out of cells is often a problem. Product recovery can be troublesome, if the product is not excreted from the cells. Chiral transport can lead to problems if racemic substrates are used. Cells often contain several dehydrogenases with opposite stereoselectivity (“iso”-enzymes) which compete for the same substrate. As a result, the stereoselectivity is reduced. SHIEH (1987) and NAKAMURA et al. (1991) demonstrated for a β-ketoacid ester that two (S)- and two (R)-specific dehydrogenases are present in bakers’ yeast. Depending on the origin and cultivation conditions of the cells, variations in yield (10–85%) and enantioselectivity (40–98%) were observed in the case of reductions of β-ketoacid esters with bakers’ yeast (POPPE and NOVAK, 1992; CHRISTEN and CROUT, 1987; WARD and YOUNG, 1990; BUSSON et al., 1992). Moreover, the substrate and/or product may be metabolized resulting in reduced overall yields.

The productivity of microbial conversions is usually low since the majority of nonnatural substrates are toxic to the living cell and are therefore tolerated only at low concentrations (0.1–0.3%). If the electron transport chain is not involved in a reduction reaction, one possibility to avoid the toxicity of substrates or products is to work with resting cells. However, the large amount of biomass present in the reaction mixture reduces the overall yield and often makes product recovery problematic.

Due to a high excess of glucose or sucrose added as auxiliary substrates for coenzyme regeneration, by-products are formed which often impede product purification. Only a small amount (typically 0.5–2%) of the carbon source is involved in coenzyme regeneration.

The solvent selection for whole-cell biotransformations in organic media is problematic, since for each pair of solvent and organism a specific cytotoxicity is found (SALTER and KELL, 1995). The well-established log P concept (the octanol: water partition coefficient; LAANE et al., 1987) for enzymatic conversions does not correlate with the cytotoxicity of organic solvents. Thus, mixtures of solvents optimized for a given organism have to be used in microbial conversions.

The above described aspects of whole-cell biotransformations are summarized in Tab. 2 and are compared with enzymatic systems. The main advantages of microbial over enzymatic conversions are the low price of the catalyst and the avoidance of cofactor recycling. On the other hand, enzymatic systems do have considerable advantages since they are well defined, controllable, scalable, and can be operated continuously.

### 3 Regeneration of Nicotinamide Coenzymes

#### 3.1 Introduction

Nicotinamide adenine dinucleotide (NAD, 6a) and the analogous 2’-phosphate (NADP, 6b) (Fig. 2) are involved in redox reactions catalyzed by alcohol dehydrogenases. Two electrons and a proton (hydride) are transferred from the reduced coenzyme NAD(P)(H) to the carbonyl compound in a stoichiometric reaction. Using coenzyme recycling, the expensive cofactor is needed only in catalytic amounts leading to a drastic reduction in cost of dehy-