1
Biooxidation with PQQ- and FAD-Dependent Dehydrogenases
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Summary

Among the obligate aerobic bacteria, acetic acid bacteria are well known for their powerful ability to oxidize alcohols, sugars, or sugar alcohols and to accumulate the corresponding oxidation products in the culture medium. These reactions are restricted to one-step incomplete oxidation (so-called oxidative fermentation) and are catalyzed by primary dehydrogenases located on the outer surface of the cytoplasmic membrane, the active sites of which face the periplasmic space. All enzyme activities are linked, without exception, to the terminal ubiquinol oxidase via ubiquinone in the respiratory chain of the organisms. The respective primary dehydrogenases working in the periplasmic sugar and alcohol respirations include many unique pyrroloquinoline quinone (PQQ)-dependent dehydrogenases (quinoproteins and quinoprotein–cytochrome c complexes) and flavin adenine dinucleotide (FAD)-dependent dehydrogenases (flavoprotein–cytochrome c complexes). Since this sugar and alcohol respiration does not seem to generate much energy, acetic acid bacteria use rapid oxidation to produce a large number of oxidation products, compensating for the necessary bioenergy required.

We have learnt a lot about the biological activities of acetic acid bacteria in the past hundred years. Among them are classic but typically important microbial bioconversions for practical use, such as the production of vinegar, D-gluconate, and L-sorbose. However, our understanding of the molecular mechanisms remains to be clarified. We have been trying to uncover the enzymatic and biochemical mechanisms of the respective enzymes in acetic acid bacteria since the 1970s. In this chapter, the properties and characteristics of the individual enzymes involved in oxidative fermentation are exemplified.

1.1
Introduction

Many membrane-bound dehydrogenases in the periplasmic space or on the outer surface of the cytoplasmic membrane of acetic acid bacteria and other aerobic
Gram-negative bacteria have been classified as PQQ- or FAD-dependent dehydrogenases [1]. Most of the enzymes are closely associated with oxidative fermentation in industry, catalyzing an incomplete one-step oxidation, allowing accumulation of an equivalent amount of corresponding oxidation products outside the cells. The active sites of individual enzymes face the periplasmic space as illustrated in Fig. 1.1.

All enzyme reactions are carried out by periplasmic oxidase systems, including alcohol- and sugar-oxidizing enzymes of the organisms. D-Glucose, ethanol, and many other substrates are oxidized by the dehydrogenases (shown as PQQ or FAD, except for aldehyde dehydrogenase) that are tightly bound to the outer surface of the cytoplasmic membranes of the organism. These membrane-bound enzymes irreversibly catalyze incomplete one-step oxidation and the corresponding oxidation products accumulate rapidly in the culture medium or reaction mixture. The electrons ($e^-$) generated by the action of these dehydrogenases are transferred to ubiquinone in the membrane. The reducing equivalents are further transferred to the terminal ubiquinol oxidase in the cytoplasmic membranes. Thus, the organisms generate bioenergy through the enzyme activities of PQQ- and FAD-dependent dehydrogenases. The outer membrane of the organism forming the periplasmic space is omitted from Fig. 1.1. Many different NAD- or NADP-dependent dehydrogenases in the cytoplasm have no function in oxidative fermentation, and thus are not shown in Fig. 1.1.

![Fig. 1.1 Membrane-bound PQQ- and FAD-dependent primary dehydrogenases on the outer surface of acetic acid bacteria.](image-url)
At present, of the enzymes exploited as either PQQ-dependent or FAD-dependent dehydrogenases, aldehyde dehydrogenase is the only one that is known to use a molybdopterin coenzyme. Unlike the cytoplasmic oxidoreductases, no energy is required for substrate intake into the periplasm and pumping out the oxidation products across the outer membrane as shown in Fig. 1.2.

Microbial production of L-sorbose, aldehyde, and 2-keto-D-glucuronate are the examples shown in Fig. 1.2. All substrates are oxidized by the respective membrane-bound dehydrogenase, of which active site faces the periplasmic space formed between the outer membrane and the cytoplasmic membrane. The dehydrogenase then donates electrons to ubiquinone (UQ) that in turn transfers them to the terminal ubiquinol oxidase. The terminal oxidase generates an electrochemical proton gradient either by charge separation or by a proton pump or by both during substrate oxidation by the membrane-bound enzymes, allowing the organism to acquire bioenergy through substrate oxidation.

Traditionally, acetate fermentation (vinegar production) and L-sorbose fermentation are typical examples of oxidative fermentation and the classic case of microbial bioconversion. Our understanding of the mechanism of oxidative fermentation, however, was not elucidated until relatively recently. Most enzymes involved in oxidative fermentation are associated closely with industrial applications for useful biomaterial production. The production of acetate, L-sorbose, D-glucuronate, dihydroxyacetone, and others developed as a practical industry before the clarification of the molecular mechanisms of the responsible enzymes. It was in 1970s that we started to clarify the molecular mechanisms of the individual enzymes involved in oxidative fermentation.

Before describing the actions of the individual PQQ- and FAD-dependent dehydrogenases, it is worth clarifying the common physiological roles and localizations of PQQ- and FAD-dependent dehydrogenases in acetic acid bacteria and
Biooxidation with PQQ- and FAD-Dependent Dehydrogenases

Other microorganisms. Many people still believe that acetate is produced by the cytosolic NAD(P)-dependent alcohol dehydrogenase and keto-D-gluconate by the cytosolic NADP-dependent D-gluconate dehydrogenase located in the cytoplasm. Such a serious confusion is probably caused by the confused localization of the enzymes concerned. Both membrane-bound enzymes and NAD(P)-dependent enzymes sometimes occur in the same cell-free extract when bacterial cells are broken down and the cell-free extract is prepared. Some periplasmic enzymes, such as quinoprotein methanol dehydrogenase in methylotrophs, are readily solubilized when the cell-free extract is prepared [2]. Given that oxidative fermentation is only functional under fairly acidic conditions, D-gluconate oxidation with an NADP-dependent enzyme observed at alkaline pH is unlikely to participate directly in keto-D-gluconate production under acidic conditions.

Although FAD is linked covalently to FAD-dependent enzymes and PQQ is tightly bound to enzyme proteins (though all PQQ-dependent enzymes (quinoproteins) contain PQQ as dissociable form), most of the membrane-bound dehydrogenases indicated earlier were stable and active without exogenous addition of the responsible coenzyme, giving the impression that they were coenzyme-independent or NAD(P)-independent dehydrogenases. However, when the cell-free extract is centrifuged under stronger centrifugal force (e.g. 68000 × g for 60 min), the enzyme activity of the membrane-bound enzymes precipitates as the membrane fraction, while the majority of NAD(P)-dependent enzymes exist in the supernatant. A typical membrane-bound dehydrogenase can be freed from NAD(P)-dependent enzyme activity by such simple fractionation of cell-free extract. The membrane-bound dehydrogenases are only solubilized by the aid of detergents. Addition of a chaotropic agent like KCl sometimes increases the recovery of solubilized enzyme.

According to the physiological roles of membrane-bound and periplasmic enzymes, it should be beneficial to detoxify cellular toxic compounds such as methanol or amines outside cells (for example, in the periplasmic space) and not in the cytoplasm. When a cell-free extract is fractionated with ammonium sulfate, membrane-bound dehydrogenases tend to precipitate at relatively low concentrations of ammonium sulfate, in contrast to NAD(P)-dependent enzymes. Sometimes unexpectedly low enzyme recovery is seen after purification of cell-free extracts on ion exchange chromatographic column, suggesting the presence of membrane-bound enzymes.

1.2
Basic Technical Information Regarding Membrane-bound Enzymes

1.2.1
Preparation of Cytosolic Fractions and Membrane Fractions

A cell suspension is prepared by homogenizing freshly harvested cells at a ratio of about 10g wet cells per 10mL of appropriate buffer solution. For mechanical
cell disruption, a cell suspension is passed through a French pressure cell press at 1000 kg cm\(^{-2}\) or other criteria. After removal of the intact cells by a conventional low-speed centrifuge, the crude cell-free extract is further centrifuged at 68000 \(\times g\) for 60 min to separate the membrane fraction as precipitate, and the resulting supernatant is designated as the soluble fraction. In the soluble fraction, it should be noted that enzyme activities of both cytoplasmic and periplasmic enzymes are usually observed. It is better to wash the resulting membrane precipitate by suspending and homogenizing in the same buffer by ultracentrifugation one or two times to eliminate the soluble enzymes.

1.2.2
EDTA Treatment of the Membrane Fraction Carrying PQQ as Coenzyme

After a membrane suspension (10 mg of protein per mL) is mixed with 20 mmol L\(^{-1}\) EDTA for 30 min in an ice bath. The excess EDTA is removed from the membrane by ultracentrifugation two or three times at 68000 \(\times g\) for 60 min. The precipitate is resuspended in a buffer to wash EDTA out of the membrane fraction, followed by ultracentrifugation again under the same conditions. The resulting precipitate is resuspended with the same buffer. Under these conditions, as mentioned below, many PQQ-dependent dehydrogenases are resolved to apoenzymes. However, some PQQ-dependent dehydrogenases are still active, although some decrease in enzyme activity is observed. If no loss of enzyme activity is found, the presence of covalently bound FAD as the coenzyme is the alternative possibility. To convert the apoenzyme to the holoenzyme, PQQ and divalent cations such as Ca\(^{2+}\) or Mg\(^{2+}\) are added to 5 \(\mu\)mol L\(^{-1}\) and 5 mmol L\(^{-1}\), respectively, and the enzyme incubated, for example, for 30 min at 25ºC, until full enzyme activity returns.

1.2.3
Assays of Enzyme Activity

Most PQQ- and FAD-dependent dehydrogenases can be assayed using artificial electron acceptors such as potassium ferricyanide or phenazine methosulfate (PMS) [3]. In the case of potassium ferricyanide, enzyme activity can be assayed over a broad pH range, from highly acidic to highly alkaline conditions. On the other hand, enzyme activity measurement with PMS combined with dichlorophenol indophenol (DCIP) is invalid at acidic pH below 6 due to non-enzymatic decolorization of the electron acceptor used. Thus, the assay with PMS-DCIP is valid in the neutral to alkaline regions.

It is also worth noting that when enzymes containing a heme \(c\) component in the enzyme molecule or membrane fraction are used, the enzyme activities can be easily assayed with potassium ferricyanide. However, enzyme activity measurement with PMS-DCIP is invalid if the enzymes do not contain the heme \(c\) component after solubilization from the membrane.
### 1.3 PQQ-Dependent Dehydrogenases

#### 1.3.1 Alcohol Oxidation

#### 1.3.1.1 Membrane-Bound Alcohol Dehydrogenase (ADH III)

Quinohemoprotein alcohol dehydrogenase (ADH III) is localized on the outer surface of the cytoplasmic membrane of the acetic acid bacteria, *Acetobacter* and *Gluconobacter*. ADH III has the most important role in vinegar production in oxidizing ethanol to acetaldehyde. The acetaldehyde generated is immediately oxidized to acetate by aldehyde dehydrogenase located close to ADH III on the same cytoplasmic membrane. As mentioned elsewhere, most people still believe that ethanol oxidation during vinegar production must be catalyzed by a cytosolic NAD-dependent ADH, even though the discovery of ADH III was reported in 1960s. ADH III catalyzes alcohol oxidation under acidic conditions at pH 3–4, which is a favorable biological environment for vinegar production. It is distinct from the cytosolic NAD-dependent ADH that oxidizes alcohol under fairly alkaline conditions at pH 9–11. When acetic acid bacteria are growing on ethanol, the enzymic activity of the cytosolic NAD-dependent ADH is very weak and almost undetectable.

Among a huge number of PQQ-dependent dehydrogenases, ADH III has been purified and demonstrated to be a typical example of a membrane-bound dehydrogenase [1]. It was the first membrane-bund enzyme to be crystallized (in 1982) [4]. The purified ADH III shows heme c type absorption spectra with absorption maxima at 555, 523, and 418 nm in the reduced enzyme. A shift in the γ-peak to 413 nm was observed for the oxidized enzyme. ADH III consists of three different subunits: subunit I (80kDa), subunit II (50kDa), and subunit III (20kDa) (Fig. 1.3). Subunit I contains the catalytic site involving PQQ as the primary co-enzyme and one heme c, forming a superbarrel structure surrounded by eight propeller structures based on tryptophan [5].

ADH III has been shown to donate electrons to ubiquinone embedded in the membrane phospholipids, and then to the terminal oxidase. Although no ADH III subunits have a transmembrane domain, subunit II (cytochrome subunit) has been shown to have a ubiquinone reacting site and to have two amphiphilic α-helices as a possible membrane anchor [6]. ADH III also seems to bind to the membrane and thus to transfer the reducing equivalent to ubiquinone via subunit II. Subunit II involves three heme c components and binds the ADH III to the cytoplasmic membrane [6, 7]. The physiological function of subunit III is still unknown but it is obviously important in the ADH III reaction. If subunit III is deleted, ethanol oxidation no longer takes place.
1.3 PQQ-Dependent Dehydrogenases

The ethanol oxidase system is composed of three simple components: ADH III, ubiquinone-9 (UQ₉) in the case of *Acetobacter* or ubiquinone-10 (UQ₁₀) in *Glucobacter*, and the transmembrane terminal oxidase, which functions as an ubiquinol oxidase. The electron generated is transferred to UQ₉ or UQ₁₀ converting it to the reduced form, ubiquinol-9 or ubiquinol-10, which in turn are oxidized to UQ₉ or UQ₁₀ again. The terminal oxidase transfers the reducing equivalent across the cytoplasmic membrane, yielding a proton gradient (inside negative) allowing bioenergy generation. Thus, acetic acid bacteria are able to obtain bioenergy during substrate oxidation on the outer surface of the cytoplasmic membrane. This hypothesis has been confirmed using a reconstituted proteoliposome of the alcohol oxidase system involving ADH III, UQ₉ or UQ₁₀ and the terminal oxidase [8]. A clear proton gradient across an artificial proteoliposome membrane vesicle is formed by the addition of substrate and can be followed by quenching of fluorescent dye along with alcohol oxidation. For more details, refer to [1, 5].

ADH III catalyzes the irreversible oxidation of primary alcohols except for methanol. Alcohol oxidation is conducted with different types of catalyst containing ADH III such as growing cells (vinegar production), resting cells, isolated
membrane fractions, and purified ADH III. In this context, ADH III has been use in the construction of alcohol sensors or alcohol biosensors that are able to interact directly with an electrode. Unlike the NAD(P)-dependent alcohol dehydrogenases, alcohol oxidation is catalyzed under fairly acidic conditions at pH 3–6. Ethanol-grown cells of *Acetobacter* or *Gluconobacter* show a strong ethanol-oxidizing activity with the membrane fraction while a little enzyme activity of NAD(P)-dependent alcohol dehydrogenase is observed [9].

Ethanol oxidation with an NAD(P)-dependent alcohol dehydrogenase usually shows a pH optimum under highly alkaline conditions at pH 9–11 and aldehyde reduction to alcohol favorably occurs under acidic conditions at pH 5–6. On the other hand, if ADH III is deleted by means of genetic mutation, the enzyme activity of ADH III disappears in the membrane fraction and the cytosolic NAD(P)-dependent ADH becomes predominant and the specific activity increased remarkably. Acetate production with such a mutant no longer takes place and the total cell growth increases, because ethanol added to the culture medium is fully converted to carbon and energy sources for the mutant [10].

Among strains of acetic acid bacteria, acetate overoxidation (also known as acetate peroxidation) is an unfavorable phenomenon in acetate brewery. Our understanding of the molecular mechanism and physiological aspects of acetate peroxidation have progressed [6, 11]. Structural studies of ADH III by means of X-ray crystallography are now being undertaken.

In the case of ADH III of acetic acid bacteria, direct electron transfer to the electrode has been shown by direct binding of the enzyme to the hydrophobic surface of a gold electrode, which is able to transfer electrons directly to the electrode without any help of electron mediator [12, 13]. Such direct electron transfer to the electrode with ADH III has been confirmed in the case of the enzyme being entrapped onto a platinum electrode with polypyrrole [14]. This intermolecular electron transfer reaction has also been observed in two quinohemoproteins: ADH IIB (soluble quinohemoprotein ADH, see below) and ADH III. Based on the crystal structure of ADH IIB [15–17], it can be seen that the heme c domain in the C-terminus of the primary structure is distant from the N-terminal PQQ domain, which is highly homologous to the superbarrel structure of other PQQ-dependent enzymes [5], and covers the PQQ domain. The distance between the heme c and PQQ is about 20 Å in the reduced form, which seems to be a little far for direct electron transfer from PQQ to heme c. Therefore, the electron transfer from PQQ to heme c could occur via an amino acid backbone present between both domains, or directly, upon a conformational change due to oxidation. The same kind of electron transfer may be expected in the quinohemoprotein subunit of ADH III.

Furthermore, in the multimeric enzyme it has been suggested that electrons extracted from ethanol at the PQQ site could be transferred via the heme c site in the subunit I to one of three heme c sites in the cytochrome subunit (subunit II), and the electrons passed to ubiquinone through two of the heme c moieties (Fig. 1.3) [18, 19]. In the case of ADH III, subunit II has been shown to have a ubiquinone reacting site despite having no transmembrane domain. Thus, ubi-
quinone reduction in ADH III may occur via electron transfer from PQQ through three of the four heme c moieties present. The electron transfer kinetics of quinoproteins or quinohemoproteins may become critical from the biotechnological point of view, especially when applying them to alcohol biosensors.

1.3.1.2 **Soluble Alcohol Dehydrogenases**

Alcohol → Aldehyde

A bacterial strain that is able to utilize several kinds of alcohols as its sole carbon and energy sources was isolated from soil and tentatively identified as *Pseudomonas putida* HK5 [20]. Three distinct dye-linked ADHs, each of which contains PQQ as the prosthetic group, are formed in the periplasmic space of the strain grown on different alcohols and thus the enzyme activities appear in the cell-free extract in soluble form.

ADH I is formed most abundantly in the cells grown on ethanol and is similar to the quinoprotein ADH reported from *P. putida* [21], except for its isoelectric point. The other two ADHs, ADH IIB and ADH IIG, are formed separately in the cells grown on 1-butanol and 1,2-propanediol, respectively. Both of these enzymes contain heme c in addition to PQQ and function as quinohemoprotein dehydrogenases. Thus, potassium ferricyanide is an available electron acceptor for ADHs IIB and IIG but not for ADH I. The molecular masses are estimated to be 69 kDa for ADH IIB and 72 kDa for ADH IIG, and both enzymes have been shown to be monomers.

Antibodies raised against each of the purified ADHs could distinguish the ADHs from one another. Immunoblot analysis showed that ADH I is detected in cells grown on each alcohol tested, but ethanol was the most effective inducer. ADH IIB is formed in cells grown on alcohols of medium chain length and also on 1,3-butenediol. Induction of ADH IIG is restricted to 1,2-propanediol or glycerol, of which the former alcohol is more effective. These results from immunoblot analysis correlate well with the substrate specificities of the respective enzymes. Thus, three distinct quinoprotein ADHs are formed by a single bacterium under different growth conditions. Molecular cloning and structural analysis of ADH IIB and ADH IIG have been reported [16, 17, 22]. These soluble forms of ADHs are also used for alcohol sensors. Of the three different PQQ-dependent ADHs, ADH IIG would be the most convincing for use as a glycerol sensor for a neutral fat diagnostic test by combining with lipoprotein lipase.

1.3.1.3 **Cyclic Alcohol Dehydrogenase (Secondary Alcohol Dehydrogenase), Membrane-Bound**

Cyclic alcohol → Cyclic ketone
Aliphatic secondary alcohol → Aliphatic ketone
Numerous investigations on microbial and enzymatic oxidation of cyclic alcohols have been carried out with various bacteria. Most of them are related to NAD-dependent cyclohexanol dehydrogenase (EC 1.1.1.245) from pseudomonads and Acinetobacter sp. [23–26]. Microbial cyclopentanol oxidation was also studied in Pseudomonas sp. and NAD-dependent cyclopentanol dehydrogenase (EC 1.1.1.163) was indicated as the responsible enzyme in the first step of the oxidative metabolism of cyclic alcohol [27]. Cyclohexanol oxidation was also observed by a secondary alcohol oxidase from Pseudomonas sp. in which hydrogen peroxide-producing flavin-dependent enzyme is functional [28–30]. The microorganisms described in the abovementioned papers do not accumulate oxidation products outside the cells, because most enzymes dealt with are cytoplasmic enzymes. Nothing has been reported, therefore, about membrane-bound cyclic alcohol dehydrogenase located on the outer surface of the cytoplasmic membrane allowing accumulation of the oxidation products outside the cells.

A quinoprotein catalyzing oxidation of cyclic alcohols was found in the membrane fraction of acetic acid bacteria. After extensive screening, Gluconobacter frateurii CHM 9 was selected. EDTA treatment with the membrane fraction indicated that the membrane-bound cyclic alcohol dehydrogenase is a PQQ-dependent dehydrogenase. From the membrane fraction, PQQ-dependent cyclic alcohol dehydrogenase was purified [31]. In contrast, from the cytoplasmic fraction of the same organism, an NAD-dependent cyclic alcohol dehydrogenase was purified and crystallized. The substrate specificities of the two differently localized enzymes showed an interesting contrast with each other, suggestive of their different physiological roles in the organisms. Unlike the already known cytosolic NAD(P)H-dependent alcohol-aldehyde or alcohol-ketone oxidoreductases, the PQQ-dependent enzyme is unable to catalyze reduction of cyclic ketones or aliphatic ketones to cyclic alcohols or aliphatic secondary alcohols.

PQQ-dependent cyclic alcohol dehydrogenase was solubilized from the membrane fraction by differential solubilization to eliminate the predominantly existing ADH III and the two subsequent steps of column chromatography gave a homogeneous enzyme preparation. The purified PQQ-dependent enzyme has a molecular mass of 83 kDa in SDS-PAGE. A wide variety of cyclic alcohols are oxidized with the PQQ-dependent enzyme. Once the enzyme was solubilized, only the PMS-DCIP assay was valid, because the enzyme does not contain any heme c component. When the PQQ-dependent enzyme was compared with the cytosolic NAD-dependent enzyme, the reaction rate and the total enzyme activity found in cyclic alcohol oxidation with the PQQ-dependent enzyme was more than 100 times higher than that with the cytosolic NAD-dependent enzyme.

The NAD-dependent enzyme is unfavorable for oxidation reactions, but is favorable for the reduction of cyclic ketones to cyclic alcohols or aliphatic ketones to aliphatic secondary alcohols. Thus, the NAD-dependent enzyme makes no contribution to cyclic alcohol oxidation, and reduction of cyclic ketones and aliphatic ketones to their corresponding alcohols is favored in the presence of NADH.
1.3 PQQ-Dependent Dehydrogenases

1.3.2 Glucose Oxidation

1.3.2.1 Membrane-Bound d-Glucose Dehydrogenase (m-GDH)

\[
\text{D-Glucose} \rightarrow \text{D-glucono-δ-lactone}
\]

The quinoprotein D-glucose dehydrogenase (EC 1.1.99.17) (GDH) occurs on the outer surface of the cytoplasmic membrane of oxidative bacteria such as *Pseudomonas* and *Gluconobacter* strains and catalyzes direct oxidation of D-glucose to D-gluconate via D-glucono-δ-lactone (membrane-bound D-glucose dehydrogenase, m-GDH). It is known as an alternative pathway to the phosphotransferase system of bacteria to catalyze D-glucose assimilation. m-GDH is found in a variety of bacteria including Gram-negative facultative anaerobes such as enteric bacteria and *Zymomonas*, as well as aerobic bacteria such as pseudomonads and acetic acid bacteria.

GDH was originally investigated in *Acinetobacter calcoaceticus* in the early 1960s by Hauge [32–35] and subsequently in the late 1970s by Duine et al. [36], who showed the enzyme to be a quinoprotein. *A. calcoaceticus* contains a soluble form of GDH (s-GDH) in addition to a membrane-bound form (m-GDH). Thus, they had been believed for many years to be the same enzyme or interconvertible forms. However, recent evidence has shown that this soluble enzyme is not a typical m-GDH. The membranes from several strains such as *Escherichia coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Gluconobacter suboxydans*, *Acetobacter aceti*, and *A. calcoaceticus* contain antigens cross-reactive with an antibody of m-GDH purified from *P. fluorescens* [37], while s-GDH purified from the soluble fraction of *A. calcoaceticus* does not cross-react with the antibody [38]. Subsequently, s-GDH and m-GDH were purified separately from *A. calcoaceticus* and shown to be distinctive in all aspects, including optimum pH, kinetics, substrate specificity, ubiquinone reactivity, molecular size, and immunoreactivity [39]. As described below, s-GDH of *A. calcoaceticus* is a monomer consisting of a single polypeptide of 48–55 kDa containing one molecule of PQQ.

When m-GDH was solubilized and purified from *Gluconobacter suboxydans* IFO 12528, other species of membrane-bound dehydrogenases were eliminated by treating the enzyme solution at pH 2.5 in the initial stage of enzyme purification. The purified m-GDH was homogeneous in analytical ultracentrifugation (4.2 S) and sucrose density gradient centrifugation [40]. m-GDH from acetic acid bacteria was highly hydrophobic and 87 kDa of its molecular mass has been determined by SDS-PAGE in the presence of urea. The existence of PQQ as the primary coenzyme has been confirmed with the purified enzyme. The optimum pH of D-glucose oxidation is found to be pH 3.0 with potassium ferricyanide and pH 6.0 with PMS-DCIP. The substrate specificity of the enzyme seems to be restricted to D-glucose, and other sugars are not oxidized except for maltose, which is oxidized at a low rate. Due to the hydrophobicity, the enzyme is regarded as a typical integral membrane protein in acetic acid bacteria.
Since *E. coli* does not have the PQQ gene, the bacterium produces m-GDH in the apo-form. The apoenzyme was converted to active holoenzyme by the addition of PQQ and Mg$^{2+}$ [41]. In contrast to the m-GDH found in acetic acid bacteria [40], PMS-DCIP is a convenient electron acceptor for measuring D-glucose oxidation by this enzyme. The enzyme is a typical membrane-bound enzyme highly embedded in the cytoplasmic membrane, with five membrane-spanning domains [42, 43]. The active site of m-GDH faces the periplasmic space. m-GDH was solubilized from the cytoplasmic membrane with the aid of detergent and further purified to a homogeneous state. The substrate specificity of *E. coli* m-GDH looks similar to that of *A. calcoaceticus* [39].

D-Gluconate is an important substance for the food industry as a food additive. Ca-D-gluconate is widely used as a metal polish in the lens industry. In aerobic Gram-negative bacteria including acetic acid bacteria, D-gluconate is produced from D-glucose by the action of m-GDH. It is distinct from a fungal D-glucose oxidase producing D-gluconate that the bacterial enzyme does not link to molecular oxygen directly while the fungal enzyme utilizes molecular oxygen-producing hydrogen peroxide. m-GDH has been shown to donate electrons to ubiquinone, embedded in the membrane phospholipids, and then to the terminal oxidase [1]. Unlike the previous assumption, however, m-GDH does not seem to have a ubiquinone reacting site in the deeply embedded region of the membrane. Despite having a transmembrane domain, m-GDH has been shown to react with ubiquinone near the surface of the membrane [44]. This notion has been strengthened by the recent finding that the C-terminal half of *E. coli* m-GDH, the N-terminal transmembrane domain being deleted, maintains ubiquinone reductase activity [45].

To clarify the intermolecular electron transfer of m-GDH, quantitation and identification of ubiquinone have been done, indicating that *E. coli* m-GDH contains a tightly bound ubiquinone-8 (UQ$_8$) in its molecule. A significant increase in the EPR signal was observed following D-glucose addition in m-GDH reconstituted with PQQ and Mg$^{2+}$ after the addition of D-glucose, suggesting that bound UQ$_8$ accepts a single electron from PQQH$_2$ to generate semiquinone radicals. No such increase in the EPR signal was observed in UQ$_8$-free m-GDH under the same conditions. Moreover, a UQ$_7$ reductase assay with a ubiquinone-related inhibitor (C49) revealed different inhibition kinetics between the wild-type m-GDH and UQ$_8$-free m-GDH. It is proposed that the native m-GDH bears two ubiquinone-binding sites, one (Q$_i$) for bound UQ$_8$ in its molecule and the other (Q$_n$) for UQ$_8$ in the ubiquinone pool, and the bound UQ$_8$ in the Q$_i$ site acts as a single electron mediator in the intramolecular electron transfer in m-GDH [46]. It should be noted that there is no specific D-xylose dehydrogenase other than m-GDH. D-Xylose oxidation is done by m-GDH.

### 1.3.2.2 Soluble D-Glucose Dehydrogenase (s-GDH)

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D\text{-Glucose} \rightarrow D\text{-glucono-δ-lactone}
\]

As mentioned above, *A. calcoaceticus* contains s-GDH in addition to m-GDH. s-GDH purified from the soluble fraction of *A. calcoaceticus* does not cross-react
with the antibody for m-GDH [38]. Subsequently, s-GDH and m-GDH were purified separately from *A. calcoaceticus* and shown to be distinctive in all aspects including optimum pH, kinetics, substrate specificity, ubiquinone reactivity, molecular size, and immunoreactivity [39].

s-GDH of *A. calcoaceticus* is a monomer consisting of a single polypeptide of 48–55 kDa containing one PQQ molecule. A gene (*gdhB*) for the enzyme has been cloned and the gene product is estimated to be a polypeptide of 52.8 kDa that contains a 24-amino-acid signal sequence at its N-terminus and thus it is expected to become the mature protein of 50.2 kDa having no hydrophobic regions. Thus, s-GDH seems to be translocated through the cytoplasmic membrane into the periplasmic space.

s-GDH is capable of catalyzing the oxidation of disaccharides, lactose, or maltose, as well as D-glucose. The enzyme is able to donate electrons to several artificial dyes, including PMS, DCIP as well as short-chain ubiquinone homologs, Q1 and Q2, but it is unable to react with the longer chain ubiquinones Q8 and Q9 [39]. s-GDH was purified from the soluble fraction of *A. calcoaceticus* with the overall recovery of 28% and showed a final specific activity of 2210 units mg\(^{-1}\). The substrate specificity of s-GDH is different from that of m-GDH. s-GDH oxidizes D-glucose (100%), D-fucose (28%), D-xylose (15%), D-galactose (30%), maltose (93%), and lactose (72%), while m-GDH is less reactive with disaccharides: D-glucose (100%), D-fucose (119%), D-xylose (81%), D-galactose (73%), D-ribose (54%), maltose (13%), and lactose (5%).

### 1.3.2.3 Applications of Quinoprotein GDHs as D-Glucose Sensors

As our understanding of the mechanisms of catalysis and electron transfer of quinoproteins progresses, the idea of the application of these enzymes as biosensors becomes more attractive, and thus electrochemical studies aimed at the design of specific quinoprotein-based electrodes have increased. s-GDH is the first quinoprotein applied to such a biosensor, and a D-glucose sensor with s-GDH in a single-use electrochemical test strip containing ferricyanide as a mediator is already on the market.

Prior to the appearance of this s-GDH sensor, a fungal flavoprotein D-glucose oxidase and NAD-dependent GDH had been used as the enzyme for D-glucose biosensors. However, biosensors based on D-glucose oxidase are sensitive to oxygen fluctuations in blood samples, while those based on NAD GDH have poor stability due to the loss of the cofactor NAD. These problems are overcome by using PQQ-dependent GDH, which is oxygen insensitive and has a tightly bound PQQ. In addition, since the electron transfer rate of s-GDH is very much higher than that of D-glucose oxidase, the biosensor based on s-GDH has produced more than twice the current density of similar D-glucose oxidase-based electrodes when s-GDH is immobilized in the presence of an insoluble ferrocene [47].

Thus, several characteristics of the s-GDH-based D-glucose sensor, especially the high catalytic activity and oxygen insensitivity, make it suitable for *in vivo* blood D-glucose monitoring in the management of diabetes.

Although it has still not been applied to commercial sensors, m-GDH has been examined for its electrochemical reaction on electrodes. Attempts have been
made to attach m-GDH from *Erwinia* sp. to an electrode surface using several different materials such as carbon paste, where no direct electron transfer from the enzyme is observed unless some insoluble quinone mediator is included in the paste [48]. Anodic current has been detected with *Erwinia* m-GDH depending on D-glucose concentrations, and is greatly increased in the presence of soluble electron mediators such as PMS. In order to improve the specificity and the response range toward D-glucose, the same type of m-GDH-based electrode has been prepared by immobilizing m-GDH from an *E. coli* mutant, in which His775 is substituted with Asp, on the carbon paste electrode [49]. The mutated m-GDH-entrapped electrode exhibits an expanded response range for D-glucose (3–70 mmol L$^{-1}$) and a narrower substrate specificity.

The substrate specificity of m-GDH from *G. suboxydans* IFO 12528 is attractive for D-glucose biosensors. The enzyme is highly specific to D-glucose and maltose is oxidized by only 5% of that for D-glucose. Other aldohexoses and aldopentoses have no effects on m-GDH from acetic acid bacteria [40].

1.3.3 Polyol Oxidation

1.3.3.1 D-Arabitol Dehydrogenase, Membrane-Bound

\[ \text{D-Arabitol} \rightarrow \text{D-xylulose} \]

Because there is little information about C-5 sugar alcohol oxidation, evidence has been presented confirming that l-ribulose formation, the oxidation product of ribitol, was catalyzed by the action of a membrane-bound PQQ-dependent ribitol dehydrogenase, but not by a cytosolic NAD-dependent ribitol dehydrogenase [50]. Due to the high hydrophobicity and instability of the enzyme for pentitol oxidation, its solubilization from the membrane and purification remained to be achieved.

Several physiological and catalytic properties of the purified membrane-bound D-arabitol dehydrogenase (ARDH) have been examined [51]. Solubilization of ARDH from the membrane of *Gluconobacter suboxydans* IFO 3257 was done successfully with Mydol 10. Selection of a favorable detergent, keeping ARDH as the holoenzyme during all the purification steps by the presence of PQQ and Ca$^{2+}$, and using a buffer system involving acetate buffer supplemented with Ca$^{2+}$ were necessary to treat the highly hydrophobic and thus labile enzyme. Purification of ARDH was successful after two steps of column chromatography on DEAE-Toyopearl and CM-Toyopearl in the presence of detergent and Ca$^{2+}$. The purified ARDH was homogeneous and showed a single sedimentation peak in analytical ultracentrifugation at 3.6 S of the apparent sedimentation constant.

Upon SDS-PAGE, ARDH dissociated into two different subunits of 82 kDa (subunit I) and 14 kDa (subunit II), forming a heterodimeric structure. It contained no heme component, unlike ADH IIB, ADH IIG, and ADH III. ARDH did not react with primary alcohols. The enzyme was proved to be a quinoprotein and
dissociation of PQQ was detected by HPLC by SDS-treated ARDH. PQQ and ubiquinone-10 (UQ10) were detected in a purified ARDH when enzyme solubilization was done with dodecyl-β-maltoside instead of Triton X-100. More importantly, when the membrane fraction was treated with 20 mmol L$^{-1}$ EDTA overnight, ARDH activity was lost but the enzyme activity was restored to its original level by the subsequent addition of PQQ and Ca$^{2+}$.

ARDH from *G. suboxydans* IFO 3257 was found to be a versatile enzyme for the oxidation of various sugar alcohols to the corresponding oxidation products, such as glycerol to dihydroxyacetone, ribitol to L-ribulose, D-arabitol to D-xylulose, D-sorbitol to L-sorbose, D-mannitol to D-fructose, and, surprisingly, D-gluconate to 5-keto-D-gluconate, according to the Bertrand–Hudson’s rule [52] (Fig. 1.4).

Membrane-bound glycerol dehydrogenase (GLDH) from *Gluconobacter industrius* IFO 3260 was reported to have the same wide substrate specificity as ARDH [53]. However, due to the hydrophobicity, GLDH was unstable when purified and thus insufficient data were obtained to compare it with other PQQ-dependent dehydrogenases, although GLDH has been proved to be an enzyme containing PQQ as the coenzyme. ARDH is very similar to GLDH as well as the membrane-bound quinoprotein D-sorbitol dehydrogenase (SLDH) from *G. suboxydans* IFO 3255 [54] in the following respects: broad substrate specificity to sugar alcohols, absence of heme component in the enzyme, and a molecular mass of 75–80 kDa in SDS-PAGE. It is worth noting that the enzyme catalyzes D-gluconate oxidation to yield 5-keto-D-gluconate, whereas 2-keto-D-gluconate is produced by an FAD-dependent D-gluconate dehydrogenase (GADH).

The oxidation product of D-gluconate with ARDH was identified as 5-keto-D-gluconate, when purified ARDH was used for cyclic oxidation of D-gluconate in the presence of quinol oxidase and ubiquinone Q$_{1}$ as illustrated in Fig. 1.5.

![Fig. 1.4 Bertrand–Hudson's rule in polyol oxidation.](image)
reaction mixture was only reactive with 5-keto-D-gluconate reductase [55] but not with 2-keto-D-gluconate reductase [56], and a stoichiometric amount of 5-keto-D-gluconate was detected in the reaction mixture. Thus, it was concluded that oxidative fermentation of 5-keto-D-gluconate is catalyzed by polyalcohol dehydrogenase and there is no specific 5-keto-D-gluconate-yielding D-gluconate dehydrogenase. Of course, in contrast to what had previously been believed, a cytosolic 5-keto-D-gluconate reductase cannot possibly contribute to the oxidative fermentation producing 5-keto-D-gluconate.

Regarding the enzyme structure, ARDH may be composed of two different subunits. The presence of a small open reading frame, which is very similar to the transmembrane region of m-GDH in *E. coli*, has been shown to be essential for the expression of active SLDH (Hoshino et al., personal communication). Therefore, as in SLDH, subunit II may be buried in the cytoplasmic membrane anchoring ARDH to the outer surface of the cytoplasmic membrane. Because the purified ARDH had a relatively high Q$_2$ reductase activity of 12.3 units per mg of protein, ARDH seemed to be attached to the cytoplasmic membranes in vivo and to link to their electron transfer chain via ubiquinone (Adachi et al., unpublished data). In acetic acid bacteria, sugar alcohol oxidation by the membrane-bound enzyme is in accordance with Betrand–Hudson’s rule (Fig. 1.4), that is to say, according to the generalization by Bertrand, the most favorable configuration for oxidation has the erythro form and R-configuration of two secondary hydroxyl groups adjacent to the primary alcohol group. ARDH oxidized some secondary alcohols, which must have an R-configuration.

### 1.3.3.2 *meso*-Erythritol Oxidation Dehydrogenase, Membrane-Bound

*meso*-Erythritol → L-erythrulose

There is some information about the oxidative fermentation of C$_4$ sugar alcohols in the literature, except earlier reports about *meso*-erythritol oxidation by acetic acid bacteria [57, 58]. Because L-erythrulose is not readily available from commercial sources, it is important to investigate the fermentation profile of L-erythrulose production for the identification of the enzyme responsible for *meso*-erythritol oxidation, and to purify and characterize the enzyme to provide basic information on L-erythrulose production. According to the recommendation of the US Food and Drug Administration (FDA), dihydroxyacetone should be
placed by L-erythrulose in cosmetics for those who are sensitive to dihydroxyacetone.

_Gluconobacter frateurii_ CHM 43 has been screened and shows high L-erythrulose production from meso-erythritol. NAD(P)-independent enzymes catalyzing meso-erythritol oxidation from the membrane fraction and NAD(P)-dependent enzymes from the cytosolic fraction have been purified from the organism [59]. The purified enzyme from the membrane fraction was identified as a quinoprotein and is responsible for L-erythrulose production, but the NAD(P)-dependent enzyme was independent for the production of L-erythrulose. Growing cells and the membrane fraction of the strain rapidly oxidized meso-erythritol to L-erythrulose irreversibly with almost 100% recovery at 37 ºC. L-Erythrulose was also produced efficiently by the resting cells as well. The enzyme responsible for meso-erythritol oxidation was localized on the outer surface of the cytoplasmic membrane of the organism.

The prosthetic group of the enzyme was identified to be PQQ after inactivation of the enzyme by EDTA treatment and the subsequent restoration to original levels by the exogenous addition of PQQ or PQQ and CaCl₂. The enzyme was solubilized and purified to homogeneity [59]. The purified enzyme showed a single band in SDS-PAGE with a molecular mass corresponding to 80kDa. A smaller band at 10–20kDa that anchors the enzyme to the cytoplasmic membrane was not detected. The optimum pH of meso-erythritol oxidation was found to be pH 5.0. The Michaelis constant of the enzyme for meso-erythritol oxidation was found to be 25 mmol L⁻¹. The enzyme showed broad substrate specificity toward C₃–C₆ sugar alcohols in which the erythro form of two hydroxy groups existed adjacent to the primary alcohol group according to the Bertrand–Hudson rule (Fig. 1.4).

NAD(P)-dependent meso-erythritol dehydrogenase was purified to a crystalline state. The molecular mass was estimated to be 60kDa, composed of two identical subunits. Unlike the membrane-bound quinoprotein, the enzyme catalyzes reversible oxidoreduction at an optimum pH of 9.0–10.5 for meso-erythritol oxidation and pH 6.0 for L-erythrulose reduction. It is evident that NAD(P)-dependent enzymes have no function in L-erythrulose production.

1.3.3.3 **D-Gluconate Oxidizing Polyol Dehydrogenase, Membrane-Bound**

D-Gluconate → 5-keto-D-gluconate

_Gluconobacter_ species oxidize sugars and sugar acids and uniquely accumulate two different keto-D-gluconates, 2-keto-D-gluconate, and 5-keto-D-gluconate, in the culture medium by the oxidation of D-gluconate [51, 60, 61]. Recently, PQQ-dependent ARDH and SLDH have been purified from _G. suboxydans_, both of which have similar broad substrate specificity towards several different polyols. ARDH and SLDH were shown to be identical based on their immunocross-reactivity and also on gene disruption and were suggested to be the same as the previously isolated glycerol dehydrogenase (GLDH) (EC 1.1.99.22). Thus, GLDH
is the major polyol dehydrogenase involved in the oxidation of almost all sugar alcohols in \textit{Gluconobacter} \textit{sp}.

In addition, the so-called quinoprotein GLDH was also uniquely shown to oxidize D-gluconate, which was completely different from flavoprotein D-gluconate dehydrogenase (GADH) (EC 1.1.99.3), that is the direct catalyst for the production of 2-keto-D-gluconate. During the investigation of ARDH, it was found that D-arabitol oxidation was always parallel to D-gluconate oxidation. The gene disruption experiment and the reconstitution of the purified enzyme clearly showed that the production of 5-keto-D-gluconate in \textit{G. suboxydans} is solely dependent on the quinoprotein GLDH (Fig. 1.6).

Production of 5-keto-D-gluconate is important, allowing the Gray’s method for vitamin C production via L-idonate and 2-keto-L-gulonate to be practical. This pathway looks stable unlike the other well-known routes involving L-sorbose or 2,5-diketo-D-gluconate as the intermediates. 5-Keto-D-gluconate is a stable compound while L-sorbose and 2,5-diketo-D-gluconate are labile compounds (Fig. 1.7).

Exclusive 5-keto-D-gluconate production would be possible using a mutant in which GADH yielding 2-keto-D-gluconate is deleted. As new information on 5-keto-D-gluconate production accumulates, 5-keto-D-gluconate production should become practical, which will allow us to utilize the new route for vitamin C production proposed by Gray [62, 63]. According to this, half of the 5-keto-D-gluconate is converted to L-idonate and then to 2-keto-L-gulonate by another oxidative fermentation before finally being converted to L-ascorbate, as shown in Fig. 1.7.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.6}
\caption{Comparison of keto-D-gluconate accumulation with a wild strain (left frame) and a mutant lacking polyol dehydrogenase (right frame). \textit{G. suboxydans} IFO 3255 wild-type strain (left frame) and GLDH-defective mutant 3255sldA::Km (right frame) were cultured in 100 mL of D-glucose–D-gluconate medium. During cultivation, 2KGA and 5KGA were analyzed periodically as indicated.}
\end{figure}
1.3.3.4 Glycerol Dehydrogenase, Membrane-Bound

Glycerol → Dihydroxyacetone

Glycerol oxidation to dihydroxyacetone in acetic acid bacteria is catalyzed by the membrane-bound quinoprotein glycerol dehydrogenase (GLDH), synonymous to polyol dehydrogenase, which catalyzes direct oxidation of various kinds of polyols according to the Bertrand–Hudson rule (Fig. 1.4). Since Bertrand reported the oxidation of glycerol to dihydroxyacetone by a sorbose bacterium that he had isolated, the production of dihydroxyacetone by oxidative fermentation advanced [60], although the nature of the responsible enzyme for dihydroxyacetone production remained unclear.

It was in 1985 that the first investigation of the membrane-bound GLDH of *Gluconobacter industrius* IFO 3260 was carried out [53]. In most acetic acid bacteria, the enzyme activity of ADH III is predominant in the cytoplasmic membrane and GLDH is readily solubilized together with ADH III. Once GLDH is solubilized, it is quite difficult to separate GLDH from ADH III under common techniques of chromatography. Thus, for the solubilization and purification of GLDH, *G. industrius* IFO 3260 was selected, although the total enzyme activity of GLDH was not as high the strain showed the least enzyme activity of ADH III.
For dihydroxyacetone production by growing cells, strains such as *G. cerinus* IFO 3264, IFO 3268, *G. gluconicus* IFO 3171, *G. suboxydans* IFO 3286, *G. suboxydans* IFO 3291, and *G. suboxydans* IFO 12528 would give better yields of dihydroxyacetone from glycerol.

Since GLDH is highly hydrophobic and seems to be deeply embedded in the cytoplasmic membrane, the improved stability of GLDH after solubilization with detergents became the immediate subject to which a clear solution was required. Among various detergents tested, 0.5% of dimethyldecylamineoxide was chosen as a favorable detergent for GLDH solubilization and GLDH purification was done simply by fractionation with polyethylene glycol 6000 (PEG). The precipitate obtained with PEG from 15 to 25% gave 100 times increase in the specific activity from the membrane fraction, giving a final yield of 25%. The purified enzyme was transparent and had no characteristic color, unlike ADH III.

During enzyme purification, contamination by other species of membrane-bound dehydrogenases found in the same membrane fraction of *G. industrius*, such as alcohol, D-gluconate, D-fructose, and aldehyde dehydrogenases, was completely eliminated. This was checked by substrate specificity. The oxidation rate of glycerol compared with that of other polyols such as meso-erythritol, D-arabitol, or D-sorbitol settled down to a constant level throughout the purification, while the enzyme activity toward D-glucose, D-fructose, or D-gluconate disappeared.

When GLDH purification was tried, the numbers of detergents available for enzyme solubilization were restricted. If GLDH purification could be done with the other recently developed detergents such as octyl-β-glucoside, dodecyl-β-maltoside or a series of Mydol, stability would be much improved as is seen with D-arabitol dehydrogenase (ARDH) or meso-erythritol dehydrogenase.

From the results obtained with GLDH, it is highly probable that oxidation of different polyols is catalyzed in acetic acid bacteria with an enzyme like GLDH. GLDH may be used for a diagnostic sensor monitoring glycerol generated from neutral fat after hydrolysis with lipoprotein lipase. Since GLDH catalyzes irreversible oxidation of glycerol to dihydroxyacetone and there is no reaction equilibrium, even a trace amount of glycerol generated can be measured with high accuracy, unlike other NAD(P)-dependent enzymes.

### 1.3.3.5 D-Mannitol Dehydrogenase, Membrane-Bound

\[
\text{D-Mannitol} \rightarrow \text{D-fructose}
\]

D-Mannitol oxidation to D-fructose in acetic acid bacteria is catalyzed by the membrane-bound PQQ-dependent polyol dehydrogenase, similar to GLDH or ARDH. The oxidation of D-mannitol to D-fructose was reported in many ketogenic acetic acid bacteria, as in the case of D-sorbitol oxidation. Fulmer and Underkofler looked at the possibility of using this oxidation for the industrial production of D-fructose using *A. suboxydans* (*G. suboxydans* at present) [64]. More detailed study of polyol dehydrogenase from acetic acid bacteria was carried out by Arcus and
1.3 PQQ-Dependent Dehydrogenases

Two different enzymes were indicated in the organism. One enzyme was an NAD(P)-independent particulate dehydrogenase active on D-mannitol and other polyols at pH 5.0, with a substrate specificity following Bertrand–Hudson’s rule (Fig. 1.4). The other enzyme was NAD(P)-dependent. As shown in other cases in this chapter, NAD(P)-dependent dehydrogenases have no use for oxidative fermentation. Thus, only the membrane-bound enzyme contributes to D-mannitol oxidation to D-fructose.

In our recent study, several strains of thermotolerant acetic acid bacteria that can grow at 37–40 °C were screened. Typical mesophilic strains cannot grow at these temperatures. Among the thermotolerant strains, *Gluconobacter frateurii* CHM 16 was chosen as the best biocatalyst for D-fructose production with the highest conversion efficiency as well as the highest yield [66]. Purification of the enzyme catalyzing D-mannitol oxidation was not tried, due to the issues described above. If we tried this, we would get a polyol dehydrogenase showing broad substrate specificity as seen with other examples in this chapter.

1.3.3.6 Ribitol Dehydrogenase, Membrane-Bound

Ribitol oxidation to L-ribulose in acetic acid bacteria is catalyzed by the membrane-bound PQQ-dependent polyol dehydrogenase, similar to GLDH or ARDH. Membrane-bound ribitol dehydrogenase in acetic acid bacteria catalyzes L-ribulose formation, while an NAD-dependent ribitol dehydrogenase is independent of oxidative fermentation [50]. To identify the enzyme responsible for pentitol oxidation by acetic acid bacteria, two different ribitol-oxidizing enzymes, one NAD(P)-dependent in the cytosolic fraction and the other NAD(P)-independent in the membrane fraction, were examined with respect to the oxidative fermentation. The cytoplasmic NAD-dependent ribitol dehydrogenase (EC 1.1.1.56) was crystallized from *Gluconobacter suboxydans* IFO 12528 and found to be an enzyme of molecular mass 100 kDa and 5 S as the sedimentation constant, composed of four identical subunits of 25 kDa each.

The enzyme catalyzed a shuttle reversible oxidoreduction between ribitol and D-ribulose in the presence of NAD and NADH, respectively. Xylitol and L-arabitol were well oxidized by the enzyme with reaction rates comparable to ribitol oxidation. D-Ribulose, L-ribulose, and L-xylulose were well reduced by the enzyme in the presence of NADH as cosubstrates. The optimum pH of pentitol oxidation was found to be at alkaline pH such as 9.5–10.5 and ketopentose reduction was optimum at pH 6.0.

NAD-Dependent ribitol dehydrogenase seemed to be specific to oxidoreduction between pentitols and ketopentoses. D-Sorbitol and D-mannitol were not oxidized with the NAD-dependent enzyme. However, no D-ribulose accumulation was observed outside the cells during the growth of the organism on ribitol. L-Ribulose was only accumulated in the culture medium instead, as the direct oxidation product catalyzed by the membrane-bound NAD(P)-independent ribitol
dehydrogenase. Thus, the physiological role of NAD-dependent ribitol dehydrogenase seems to be to catalyze ribitol oxidation to D-ribulose in the cytoplasm.

Phosphorylated D-ribulose is involved in the pentose phosphate pathway. L-Ribulose outside the cells could be incorporated into the cytoplasm in several ways when the use of L-ribulose as carbon and energy source becomes necessary for cell survival. From a series of simple experiment, membrane-bound PQQ-dependent sugar alcohol dehydrogenase was concluded to be the enzyme responsible for L-ribulose production from ribitol in oxidative fermentation by acetic acid bacteria.

1.3.3.7 D-Sorbitol Dehydrogenase, Membrane-Bound

D-Sorbitol → L-sorbose

D-Sorbitol oxidation to L-sorbose in acetic acid bacteria is catalyzed by the membrane-bound PQQ-dependent polyol dehydrogenase in addition to FAD-dependent D-sorbitol dehydrogenase (SLDH) (see Section 1.4.5). The introduction of D-sorbitol oxidation to L-sorbose by microbial bioconversion (L-sorbose fermentation) stimulated the vitamin C industry [60]. The chemical method for L-ascorbate synthesis developed by Leichstein in 1937 leads to racemic sorbose after oxidation of D-sorbitol, while L-sorbose fermentation gives only L-sorbose with almost 100% yield, apparently two times higher than the chemical method. Many strains of “sorbose bacterium” have been isolated and used for L-sorbose production.

Together with acetate production this process is a typical and classical microbial bioconversion with acetic acid bacteria. However, the enzymatic mechanism of L-sorbose formation remained to be clarified. In 1982, SLDH was purified for the first time from the membrane fraction of *Gluconobacter suboxydans* var. α IFO 3254 [67]. The enzyme contains a covalently bound FAD as the coenzyme, as described below (see Section 1.4.5). Until recently, before we started to survey the enzymatic properties of polyol dehydrogenases involved in D-sorbitol metabolism in acetic acid bacteria, SLDH from *G. suboxydans* var. α IFO 3254 was the sole described enzyme [50, 68, 69]. Through investigations on quinoproteins such as GLDH, ARDH, meso-erythritol dehydrogenase, and ribitol dehydrogenase, it has been concluded that these enzymes are responsible for D-sorbitol oxidation to L-sorbose as described in this chapter.

Recently, we have screened thermotolerant acetic acid bacteria that can grow at 37°C. *Gluconobacter frateurii* CHM 54 was isolated and applied to L-sorbose production [66]. In most cases, strict temperature control is required for oxidative fermentation. A hot summer readily allows indoor temperatures to rise above 30°C in many countries. This is a serious challenge not only to oxidative fermentation but also to other fermentation industries, since a huge amount of expense is required for cooling. A temperature increase by 2–3°C causes a serious failure in both fermentation rate and fermentation efficiency. In submerged cultures, a large amount of heat is generated during fermentation and cooling costs become
even more expensive. If favorable strains of acetic acid bacteria that can work optimally at 37–40°C were available, such strains would be able to accept loose temperature control and the cooling expenses would be reduced considerably.

The thermotolerant strain of *G. frateurii* CHM 54 was able to produce L-sorbose at higher temperatures. When D-sorbitol oxidation was done at higher temperatures with the thermotolerant strain, both the fermentation rate as well as fermentation efficiency were superior to that achieved using non-thermotolerant mesophilic strains [66].

1.3.3.8 **L-Sorbose Dehydrogenase, Membrane-Bound**

\[
\text{L-Sorboseone} \rightarrow \text{L-ascorbate}
\]

In current industrial L-ascorbate production processes, 2-keto-L-gulonate is a key intermediate that is chemically converted to L-ascorbate. All processes known to date require a large amount of energy and organic solvent, and thus a cheaper and environmentally conscious substitute process, such as enzymatic conversion, is being looked for. Due to the practical importance of the enzymes leading to L-ascorbate production, there are many papers dealing with the enzymes concerned (see Fig. 1.7). The following outstanding results should be itemized: isolation and characterization of a new PQQ-dependent dehydrogenase, L-sorbose/L-sorboseone dehydrogenase [70], cloning the genes coding for L-sorbose and L-sorboseone dehydrogenases from *Gluconobacter oxydans* and microbial production of 2-keto-L-gulonate, a precursor of L-ascorbate, in a recombinant *G. oxydans* [71], cloning and nucleotide sequencing of the membrane-bound L-sorboseone dehydrogenase gene of *Acetobacter liquefaciens* IFO 12258 and its expression in *Gluconobacter oxydans* [72], isolation and characterization of a new vitamin C-producing enzyme (L-gulono-γ-lactone dehydrogenase of bacterial origin [73], microbial production of L-ascorbate from D-sorbitol, L-sorbose, L-gulose, and L-sorboseone by *Ketogulonicigenium vulgare* DSM 4025 [74].

Recently, a novel PQQ-dependent enzyme, L-sorboseone dehydrogenase 1 (SNDH1) catalyzing direct conversion of L-sorboseone to L-ascorbate was purified from *Ketogluconicigenium vulgare* DSM 4025 [75]. SNDH1 is a homooligomer of 75 kDa subunits containing PQQ and heme c as the prosthetic group. Two isozymes of SNDH1, SNDH2 consisting of 75 kDa and 55 kDa subunits, and SNDH3 consisting of a 55 kDa subunit, were also purified from the same strain. It was found that the 55 kDa subunit was derived from the 75 kDa subunit after cleavage of the C-terminal domain in the bacterial cells. The three enzymes catalyzed L-ascorbic acid formation as well as 2-keto-L-gulonate from L-sorbose, suggesting that tautomerization of L-sorboseone causes the dual conversion by SNDHs. Industrial L-ascorbic acid production has revealed direct conversion of L-sorboseone to L-ascorbic acid by a membrane-bound quinoprotein L-sorboseone dehydrogenase.
1.3.4 Quinate Oxidation. Membrane-Bound Quinate Dehydrogenase (QDH)

Quinate → 3-dehydroquinate

Unlike enzymes relating to the shikimate pathway found in the cytoplasm of microorganisms, the first report of a quinate-oxidizing enzyme in acetic acid bacteria was done by Whiting and Coggins [76]. They described quinate oxidation to 3-dehydroquinate (DQA) by an NAD(P)-independent quinate dehydrogenase (QDH) (EC 1.1.99.25) and shikimate (SKA) oxidation to 3-dehydroshikimate (DSA) by an enzyme associated with the particulate enzyme in the cytoplasmic membrane. van Kleef and Duine described the occurrence of QDH in the periplasm of Acinetobacter calcoaceticus LMD 79.41 and suggested that QDH is a quinprotein in which PQQ is involved [77] (Fig. 1.8).

QDH purification was done with Gluconobacter oxydans IFO 3244 and Acinetobacter calcoaceticus AC3 after solubilization with detergent [78, 79]. Due to the hydrophobicity of QDH as a typical membrane-bound enzyme, QDH is one of the most difficult enzymes to purify to high homogeneity. The molecular mass of QDH was estimated to be 88 kDa. It oxidizes quinate and SKA with optimal activity at pH 6–7. Since A. calcoaceticus AC3 cannot produce PQQ, QDH was

![Metabolic map of shikimate pathway.](image)

**Fig. 1.8** Metabolic map of shikimate pathway.
purified as an apoenzyme consisting of dimeric structure, which was converted to the monomeric holoenzyme on addition of PQQ.

QDH is a very beneficial enzyme for the production of SKA via DQA and DSA from quinate supplied outside the SKA pathway without any metabolic control repressing SKA production. In 2003, we proposed SKA production by a single cellular system of acetic acid bacteria [80–83]. QDH and 3-dehydroquinate dehydratase (DQD) are located predominantly on the outer surface of the cytoplasmic membranes of some species of *Gluconobacter* strains and quinate is oxidized to DSA via DQA in a sequential manner. In the cytoplasm, SKDH catalyzes a reversible reaction of SKA oxidation to DSA and DSA reduction to SKA. However, many trial and error experiments to combine together the two separately located enzymatic systems taking place outside and inside the cells only resulted in insufficient production of SKA.

As a more positive response to the global need for oseltamivir, we have developed a better strategy for high SKA production, allowing the two separately located enzymatic systems to work sequentially. Dried cells or the membrane fraction involving QDH and DQD may be used for DSA production in the first reaction (system 1 in Fig. 1.9a). The second reaction is a coupling reaction composed of two cytosolic enzymes, SKDH and GDH, as an NADPH-regenerating enzyme (system 2 in Fig. 1.9a). The coupling reaction by the two cytosolic enzymes in the presence of excess D-glucose works well as expected until the DSA added initially is converted completely to SKA. The overall reaction carried out for SKA production by the two different enzymatic systems is depicted in Fig. 1.9b.

SKA is a key intermediate for aromatic amino acids as well as for large numbers of antibiotics, alkaloids, and herbicides. Recently, another important role for SKA has emerged as a precursor for the synthesis of oseltamivir (Tamiflu), an antiviral drug designed to protect people from pandemic flu infection. In spite of warnings from the World Health Organization about the approach of a global flu pandemic, including avian influenza, there are insufficient stocks of oseltamivir around the world. One reason for this is the technical difficulties in preparing SKA, because two different metabolic pathways, glycolysis and pentose phosphate pathway, need to be combined before forming SKA. Furthermore the metabolic location of SKA is a long way from that for glucose and it is very difficult to lead the metabolic flow to SKA production through classic fermentation technology as well as through modern molecular biotechnology. Although resources are limited, SKA is also produced by extraction from plants such as *Illicium anisatum* or *I. verum*. The total synthesis of SKA through organic chemistry has not been practically available. Nevertheless, we need to address the challenge of developing a novel method for more effective and convenient SKA production.

SKA is remote from D-glucose in the metabolic pathway, the more shortcut access to the SKA pathway from quinate looks advantageous to produce the important metabolic intermediates generated in the SKA pathway. DSA is formed with a yield of about 90% from quinate via DQA by two successive enzyme reactions, QDH and 3-dehydroquinate dehydratase (DQD) (EC 4.2.1.10) located in the
System 1: Oxidative fermentation for 3-dehydroshikimate formation from quinate

\[
\begin{align*}
\text{Quinate} & \rightarrow \text{3-Dehydroquinate} \rightarrow \text{3-Dehydroshikimate} \\
\text{QDH} & \rightarrow \text{DQD}
\end{align*}
\]

System 2: Conversion of 3-dehydroshikimate to shikimate by SKDH coupled with GDH

\[
\begin{align*}
\text{3-Dehydroshikimate} & \rightarrow \text{Shikimate} \\
\text{SKDH} & \rightarrow \text{GDH}
\end{align*}
\]

Fig. 1.9 (a) Overall reaction for SKA production and (b) enzymatic conversion of DSA to SKA with SKDH coupled with GDH.

cytoplasmic membranes of acetic acid bacteria. DSA is then reduced to SKA with NADP-dependent SKA dehydrogenase (SKDH) (EC 1.1.1.25) from the same organism. When SKDH is coupled with NADP-dependent glucose dehydrogenase (GDH) (EC 1.1.1.47) in the presence of excess d-glucose as an NADPH regenerating system, SKDH works to produce SKA until DSA added initially in the reaction mixture is completely converted to SKA as shown in Fig. 1.9b [83, 84].

For the system 1, the dried cells or the membrane fraction were incubated with quinate at pH 5.0 overnight with shaking. The DSA formed was measured with the deproteinized supernatants. Since DQD have a pH optimum at 8.0 and low activity of DQD is found at pH 5.0, the reaction can be regulated to make DQA as the major product. The apparent conversion rate from quinate to DQA is estimated at over 90%. On the other hand, when the above reaction was carried out
at pH 8.0, DSA was accumulated as the major product, because DQA formed can be converted immediately to DSA under the optimum condition of DQD. The apparent yield of DSA from quinate is usually over 90% [85]. Thus, a convincing strategy for preparing commercially unavailable metabolic intermediates, DQA and DSA, by means of bioconversion has been established. QDA is one of the compounds involved in the oxidative fermentation, the initially added quinate is converted to the corresponding oxidation product with high yield, as seen with other PQQ- and FAD-dependent dehydrogenases. A simple chromatographic method for separating the four compounds—quinate, DQA, DSA, and SKA—in the SKA pathway has recently become available [85].

1.4 FAD-Dependent Dehydrogenase

1.4.1 D-Fructose Dehydrogenase, Membrane-Bound

\[
\text{D-Fructose } \rightarrow \text{5-keto-D-fructose}
\]

D-Fructose oxidation to 5-keto-D-fructose (5KF) is catalyzed by membrane-bound fructose dehydrogenase (FDH, EC 1.1.99.11), which contains a covalently bound FAD as the primary coenzyme. FDH was purified from the membrane fraction of \textit{G. industrius} IFO 3260. FDH was solubilized from the membrane with 1% Triton X-100 and further purified to a homogeneous state [86]. The purified FDH had a sedimentation coefficient of 5.8S and the total molecular mass was estimated to be 140 kDa by gel filtration. The enzyme was dissociated into three different subunits, 67 kDa, 50 kDa, and 19 kDa, during gel electrophoresis. The largest subunit was positive to enzyme activity staining in a mixture containing PMS, nitroblue tetrazolium, and D-fructose.

When an unstained gel was irradiated with a fluorescent light, intense fluorescence was observed only with the largest subunit, indicating the presence of a covalently bound FAD as the coenzyme. The second subunit (50 kDa) was characterized as the subunit carrying cytochrome c. Regarding the smallest subunit, as with ADH III, GADH, KGDH, and other membrane-bound dehydrogenases, the actual function and properties of the subunit were unknown. However, if the smallest subunit is deleted, the enzyme activity of the individual enzymes is also halted and no accumulation of the corresponding oxidation product outside the cells is observed. Thus, the smallest subunit is essential to enzyme activity as well as to the oxidative fermentation.

It should be noted that only D-fructose was oxidized by FDH during the study of substrate specificity with various substrates. When D-fructose was oxidized in the presence of the same concentration of substrate analogs such as D-glucose, D-mannose, D-fructose-6-phosphate, D-fructose-1,6-diphosphate, 5-keto-D-fructose, D-glucose-6-phosphate, D-glucose-1-phosphate, D-gluconate, 2-keto-D-
gluconate, and 5-keto-D-gluconate, the reaction rate of D-fructose oxidation was not affected at all. These properties of FDH suggest that FDH could be beneficial as the enzyme for D-fructose microdetermination. As mentioned below, an apparent Michaelis constant determined at pH 4.5 was found to be 10 mmol L\(^{-1}\). The optimum pH for D-fructose oxidation was found at pH 4.0–4.5. The reaction product of D-fructose oxidation was identified to be 5-keto-D-fructose, which has no negative effect on D-fructose oxidation.

As has already mentioned above, the most characteristic point of oxidative fermentation is that D-fructose oxidation by FDH continues until the D-fructose added initially in the reaction mixture is oxidized completely to the reaction product; there is no reaction equilibrium, unlike NAD(P)-dependent dehydrogenases. With respect to the enzymatic method for D-fructose determination, a coupling enzymatic method involving hexokinase (EC 2.7.11), phospho-D-glucose isomerase (D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9), and D-glucose-6-phosphate dehydrogenase (EC 1.1.1.49) is the sole reliable method so far reported [87]. However, this assay method depends largely on the purity of the enzymes used and therefore is rather expensive. Difficulty in preparing the required enzymes without any contaminants could make this assay system complicated and potentially troublesome. A trace of D-glucose present in the samples for D-fructose assay invites inevitable confusion, because hexokinases react widely with many aldoses. The preparation of highly purified D-fructokinase that reacts with only D-fructose is almost impossible. Hence, the use of FDH is highly recommended instead of the method above, because FDH reacts only with D-fructose so can be used for the rate assay as well as for the end point measurement.

In this chapter, it is emphasized that PQQ- and FAD-dependent dehydrogenases obtained from the membrane fraction of acetic acid bacteria and other aerobic bacteria are useful and convenient enzymes for biosensors monitoring ethanol, acetaldehyde, D-glucose, D-gluconate, 2-keto-D-gluconate, and so on. In the same way, FDH can be the enzyme for D-fructose measurement for clinical purposes [88]. FDH could be beneficial for agriculture, for example in fruit production to check the best timing for ripening, and in fermentation industries for process control in brewing. FDH is now available on the market.

1.4.2
D-Gluconate Dehydrogenase, Membrane-Bound

\[ \text{D-Gluconate} \rightarrow \text{2-keto-D-gluconate} \]

D-Gluconate dehydrogenase (GADH) (EC 1.1.99.3) occurs on the outer surface of cytoplasmic membranes of aerobic bacteria, such as *Pseudomonas*, *Klebsiella*, *Serratia*, and acetic acid bacteria. The enzyme activity is linked to the electron transport chain in the cytoplasmic membrane constituting a D-gluconate oxidase system [89–91].

GADH of *P. aeruginosa* shows a single protein band on native PAGE, but the enzyme preparations from *K. klebsiella* and *S. marcescens* are separated into two
protein bands [92]. The low-mobility band is yellow and fluorescent under ultravi- 
et light, and shows enzyme activity. The coenzyme functioning has been shown to be \(8\alpha\)-[\(N^1\)-histidyl]riboflavin from the largest subunit [93]. Another protein band with higher mobility is red-colored. In SDS-PAGE, enzyme preparations from four different bacterial strains dissociate into three different polypeptide bands, subunit I having 66–68 kDa, subunit II (48–52 kDa), and subunit III (22–25 kDa). The sum of the three subunits gives 130–140 kDa as the total molecular mass. GADH is a monomeric protein dispersed in the presence of 0.1% detergent such as Triton X-100, whereas the deletion of detergent allows GADH to be the dimeric enzyme. The removal of detergent from the purified enzyme causes a decrease of enzyme activity. Activation of GADH is observed with phospholipids, especially cardiolipin, in the presence of Triton X-100. Thus, GADH has a hydrophobic and phospholipid-interacting domain, and hence is a typical membrane-bound dehydrogenase.

All GADHs are highly specific for D-gluconate oxidation with \(K_m\) values of 0.3–0.8 mmol L\(^{-1}\) and show optimum pH at 4.0–5.0, when assayed with potassium ferricyanide. The following compounds are not oxidized by GADH: aldohexoses, aldopentoses, 2-keto-D-gluconate, 5-keto-D-gluconate, D-galactonate, D-mannonate, 6-phospho-D-gluconate, L-idonate, D-arabonate, and D-xylonate. Since the enzyme activity of GADH is not affected at all by the presence of high concentrations of the above compounds, GADH is a favorable enzyme for D-gluconate measurement [94]. D-Gluconate is well known as one of the natural ingredients of brewing products and is widespread in other materials as food additives. In spite of the wide distribution and usefulness of D-gluconate, an accurate method for its quantitative measurement had not been developed until GADH was proposed for the purpose. A well-known coupling enzymatic D-gluconate determination developed and used so far contains D-gluconate kinase and 6-phospho-D-gluconate dehydrogenase. However, this assay method depends largely on the purity of the enzymes used and is rather expensive. Difficulty in preparing a pure D-gluconate kinase and 6-phospho-D-gluconate dehydrogenase without any contaminants makes this assay system complicated and troublesome. A simple chemical colorimetric determination using GADH from aerobic bacteria is highly reliable method because GADH is prepared with ease and the enzyme activity is stable without appreciable loss if GADH is stored in an appropriate buffer solutions in the presence of detergent. Rate assay as well as the end point measurement for D-gluconate can be done successfully with GADH.

1.4.3 D-Hexosamine Dehydrogenase, Membrane-Bound

\[\text{D-Glucosamine} \rightarrow \text{D-glucosaminate}\]
\[\text{D-Galactosamine} \rightarrow \text{D-galactosaminate}\]
\[\text{D-Mannosamine} \rightarrow \text{D-mannosaminate}\]

In most strains of acetic acid bacteria, membrane-bound D-hexosamine dehydrogenase (tentatively named) oxidizes D-hexosamines to the corresponding D-
hexosaminates stoichiometrically. Conversion of D-hexosamines into D-hexosaminates is observed with growing cells of acetic acid bacteria and D-hexosamine is accumulated in the culture medium after D-hexosamine is exhausted. Since the responsible enzyme is accommodated on the outer surface of the cytoplasmic membrane and the enzyme activity is linked to the respiratory chain of the organisms, resting cells, dried cells, and immobilized cells of acetic acid bacteria are effective catalyst for D-hexosaminate production. D-Mannosaminate and D-galactosaminate, commercially unavailable compounds, can be prepared with ease with acetic acid bacteria. The respective three different D-hexosaminates are shown to be separated from each other by chromatography [95].

The first report of D-glucosaminate formation by acetic acid bacteria and other aerobic bacteria like pseudomonads was carried out in experiments with resting cells and D-glucosamine was indirectly suggested to be formed as the oxidation product of D-glucosamine [96]. Recently, bioconversion of D-glucosamine to D-glucosaminate by a strain of Acinetobacter sp. isolated by enrichment techniques on D-glucosamine was briefly reported [97]. Although acetic acid bacteria are different from Acinetobacter in many respects, the enzyme functioning in D-glucosamine oxidation in Acinetobacter sp. is supposed to be the same as in acetic acid bacteria. There is no information about the substrate specificity of quinoprotein D-glucose dehydrogenase (m-GDH) or whether the enzyme is capable of D-glucosamine oxidation.

The enzyme activity of D-glucosamine dehydrogenase can be measured using either a combination of PMS and DCIP assayed in 50 mmol L⁻¹ potassium phosphate, pH 7.0, or potassium ferricyanide assayed in 50 mmol L⁻¹ glycine-NaOH buffer, pH 9.0, under essentially the same conditions as described by Ameyama [3]. Identification and measurement of D-hexosaminate can be done enzymatically with D-glucosaminate dehydratase (EC 4.2.1.26) purified from the cell-free extract of P. fluorescens IFO 14808 according to Iwamoto and Imanaga [98].

When the growth profile of G. frateurii IFO 3264 with high D-hexosamine oxidase activity was examined in 0.5% D-glucosamine medium and monitored both by measuring turbidity and by direct viable cell counting, the organism survived even though cultivation was prolonged to the late stationary phase, where the D-glucosamine was exhausted and converted to D-glucosaminate [95]. Unlike the case of Acinetobacter sp. [97], it is characteristic to see that the majority of the cells of G. frateurii IFO 3264 survived over the prolonged cultivation after complete oxidation of D-glucosamine to D-glucosaminate, as demonstrated by viable cell counting throughout the cultivation. D-Glucosamine initially added to the culture medium was converted to D-glucosaminate almost stoichiometrically, indicating that the oxidation products of D-glucosamine stayed stable without any significant breakdown by the organism. This is a noticeable difference from the case of pseudomonads or other Gram-negative bacteria, in which an appreciable amount of D-glucosaminate was further assimilated, as also suggested by Takahashi and Kayamori [96].

Encouraged by the clear data on D-hexosamine oxidation as shown above, workers have examined the microbial conversion of D-mannosamine and D-
galactosamine to yield corresponding D-hexosaminates [95]. Freshly harvested cells (10 mg mL\(^{-1}\)) of G. frateurii IFO 3264 grown to the stationary phase were mixed with either D-mannosamine-HCl or D-galactosamine-HCl in 5 mmol L\(^{-1}\) potassium phosphate, pH 7.0, and the total volume was adjusted to 10 mL. The mixtures were kept at 30 ºC with shaking and the remaining D-hexosamine in the reaction mixture was checked periodically with a purified D-glucosamine dehydrogenase. D-Mannosamine oxidation took 2 h and D-galactosamine oxidation took 8 h under the above conditions. As additional new information, D-glucosaminate dehydratase was just as effective as the three different D-hexosaminates. For example, 136% and 118% of the relative reaction rate to D-glucosaminate were observed for D-mannosaminate and D-galactosaminate, respectively, when individual oxidation products were assayed with D-glucosaminate dehydratase under steady state conditions. Thus, production of D-mannosaminate and D-galactosaminate was indicated successfully for the first time, accompanied by the enzymatic identification of the individual oxidation products.

We have to mention the reason why D-hexosamine oxidizing enzyme is classified as an FAD-dependent dehydrogenase. The main reason, after solubilization and partial purification of the respective enzyme, is that potassium ferricyanide was still valid as the electron acceptor. Second, the solubilized enzyme was highly resistant to EDTA treatment indicating either that it contains a covalently bound FAD or that the PQQ binding to the enzyme is unusually strong. The final answers will be found soon.

1.4.4
2-Keto-D-gluconate Dehydrogenase, Membrane-Bound

2-Keto-D-gluconate → 2,5-diketo-D-gluconate

The enzyme catalyzing 2-ketogluconate oxidation, yielding 2,5-diketo-D-gluconate, has been characterized as a flavohemoprotein with three different subunits [99]. From the membrane fraction of Gluconobacter melanogenus IFO 3293, 2-keto-D-gluconate dehydrogenase (KGDH) is solubilized and purified with high yield to a homogeneous state examined by the criteria of electrophoresis and analytical ultracentrifugation. The purified KGDH is homogeneous in analytical ultracentrifugation, with a sedimentation coefficient of 5.9 S, and also in native-PAGE, with a single protein band. After solubilization of the enzyme performed with 2% Na-cholate and 0.2 mol L\(^{-1}\) KCl, two-step column chromatography gave a purified enzyme. The molecular mass was measured to be 133 kDa and SDS-PAGE shows the presence of three different subunits of 61 kDa (flavoprotein), 47 kDa (cytochrome c), and 25 kDa. The flavoprotein contained a covalently bound FAD [93].

Purified KGDH has a characteristic deep rose-red color due to the cytochrome component. The typical reduced cytochrome c type absorption spectrum has maxima at 554, 523, and 417 nm. The cytochrome component of the enzyme is reduced on addition of either 2-keto-D-gluconate or sodium dithionite. A
successful purification of KGDH came from the finding of fundamental differences in hydrophobicity between D-gluconate dehydrogenase (GADH) (see Section 1.4.2) and KGDH. KGDH was not solubilized in the absence of a chaotropic agent, KCl. With Brij 35 and Tween 80, even in the presence of KCl, KGDH was scarcely solubilized, but GADH was solubilized with a final recovery of 100%. Thus, the membrane fraction of *G. melanogenus* IFO 3293 was treated first with 2% Brij 35 and 0.2 mol L\(^{-1}\) KCl to remove GADH, and after centrifugation the precipitate containing KGDH was collected. The resulting membrane precipitates were suspended in 0.01 mol L\(^{-1}\) Tris-HCl, pH 8.0, and treated with Triton X-100, Na-cholate, or Na-deoxycholate for 3 h at 5 °C. KGDH was solubilized in the presence of 0.3 mol L\(^{-1}\) KCl together with 2% Triton X-100. Alternatively, solubilization of KGDH was carried out overnight with 2% Na-cholate and 0.2 mol L\(^{-1}\) KCl, which was favorable to the following purification procedure involving ammonium sulfate fractionation.

The enzyme activity of KGDH is most active at pH 4.0, and 2-keto-D-gluconate is the only substrate oxidized by the enzyme. Similar compounds such as 5-keto-D-gluconate, 2-keto-D-galactonate, and 2-keto-D-gulonate are inert. KGDH catalyzes 2-keto-D-gluconate oxidation to 2,5-diketo-D-gluconate, which is an important step of bioconversion in a novel pathway to L-ascorbate developed by Sonoyama et al. [100]. D-Glucose was first converted to Ca-2,5-diketo-D-gluconate by a mutant strain of *Erwinia* sp. with 94.5% yield after 26 h cultivation. Then, a mutant strain of *Coprynebacterium* sp. reduced 2,5-diketo-D-gluconate stereospecifically with 2,5-diketo-D-gluconate reductase to Ca-2-keto-L-gulonate with a yield of 84.5%. The occurrence of KGDH is known in strains of *Erwinia*, *Pantoea*, and *Pseudomonas*, as well as *Gluconobacter*. Ameyama and Kondo noted the importance of 2,5-diketo-D-gluconate as the precursor of D-lyxuronic acid [101].

1.4.5

**Sorbitol Dehydrogenase, Membrane-Bound**

\[
\text{Sorbitol} \rightarrow \text{L-sorbose}
\]

Unlike the ubiquitous quinoprotein polyol dehydrogenases in acetic acid bacteria, one of which catalyzes oxidation of D-sorbitol to L-sorbose, a distinct D-sorbitol dehydrogenase (SLDH) was found in the membrane fraction of *G. suboxydans* var. a IFO 3254. SLDH was solubilized from the membrane fraction and purified as a covalently bound FAD-containing dehydrogenase [67]. The best solubilization of SLDH carried out in 10 mmol L\(^{-1}\) acetate, pH 5.0, containing 1% Triton X-100, 0.1 mol L\(^{-1}\) KCl, and 0.1 mol L\(^{-1}\) D-sorbitol gave the highest recovery of more than 100%. Purification of SLDH was readily done by DEAE-cellulose and CM-cellulose chromatography. The purified SLDH showed a rose-red color due to the presence of heme \(c\) and the absorption spectrum of SLDH after reduction with sodium dithionite gave absorption maxima at 551, 522, and 417 nm in the visible region. The heme \(c\) component was not reduced rapidly by the addition of D-sorbitol, primary substrate, until coenzyme Q\(_1\) was added to the enzyme solution,
implying that ubiquinone mediates D-sorbitol oxidation, similar to the other
membrane-bound dehydrogenases exemplified in this article.

SLDH dissociated into three subunits of 63 kDa (subunit I), 51 kDa (subunit
II), and 17 kDa (subunit III). Subunit I was characterized as a flavoprotein and
FAD was bound covalently to the enzyme. Subunit II contained heme c. SLDH
catalyzes D-sorbitol oxidation to L-sorbose exclusively, which is distinct from
PQQ-dependent polyol dehydrogenases catalyzing D-sorbitol oxidation to L-
sorbose. The substrate specificity is restricted to D-sorbitol, and D-mannitol was
oxidized only by 5% of that of D-sorbitol. Other polyols, such as D-arabitol, D-iditol,
meso-erythritol, dulcitol, ribitol, and xylitol, are not oxidized by the enzyme.

1.5 Miscellaneous

1.5.1 Aldehyde Dehydrogenase, Membrane-Bound

Membrane-bound aldehyde dehydrogenase (ALDH) in acetic acid bacteria acts
on a wide range of aliphatic aldehydes except for formaldehyde. Aldehydes with
a carbon chain length of 2–4 are oxidized most rapidly with ALDH from both
genera of Acetobacter and Gluconobacter. The enzyme is localized on the outer
surface of the cytoplasmic membrane of the organisms and has a close topological
and functional relation to ADH III. Aldehyde oxidation is linked to the respiratory
chain as described for the alcohol oxidase system above. Thus, ALDH acts as
vinegar producer sequentially after ADH III in acetic acid bacteria. During alco-
hol oxidation, no aldehyde liberation is observed under normal culture conditions,
indicating that ADH III and ALDH form a multienzyme complex in the bacterial
membrane and function sequentially to produce acetate from ethanol. As for
ALDH from acetic acid bacteria, the purification and characterization of ALDH
have been done with several strains [102–106].

As summarized by Matsushita et al. [1], purified ALDHs from G. suboxydans
and A. aceti contain heme component, while ALDHs from A. polyoxogenes and A.
rancens do not contain heme c. The subunit structures and compositions from
the different sources are different. A composition of two subunits was reported,
with ALDH from G. suboxydans composed of subunit I (86 kDa) and subunit II
(55 kDa), A. polyoxogenes, subunit I (75 kDa) and subunit II (19 kDa), and A. ran-
cens, subunit I (78 kDa) and subunit II (66 kDa). ALDH from A. aceti is composed
of three subunits: subunit I (78 kDa), subunit II (45 kDa), and subunit III
(14 kDa). The subunit I contains the catalytic site involving a molybdoputerin
cofactor as the primary coenzyme.

Purified ALDHs containing heme c from G. suboxydans [102] and A. aceti [103]
are rose-red in color with absorption maxima at 551, 523, and 418 nm (reduced
enzyme) and a sole peak at 410 nm (oxidized enzyme). Since the absorption spectra of ALDH is very similar to that of ADH III, ALDH was believed to be a PQQ-dependent enzyme. However, a mutant lacking the gene encoding PQQ biosynthesis still contained active ALDH, while the enzyme activity of ADH III was completely lost, indicating that the coenzyme of ALDH is not PQQ [107]. Following cloning of an ALDH-encoding gene from *A. europaeus* the deduced amino acid sequence indicated the presence of a molybdenum–molybdopterin cytosine dinucleotide coenzyme [108]. Molybdenum–molybdopterin cytosine dinucleotide was also indicated as the cofactor of isoquinoline 1-oxidoreductase of *Pseudomonas diminuta* 7 [109]. ALDH may have a similar coenzyme structure, although the final characterization of the coenzyme in ALDH has not been completed.

ALDH from *A. aceti* is highly stable in acidic pH as well as highly resistant to heating and more than 50% of aldehyde oxidase in the membrane fraction survived when heated at 60°C for 30 min, while enzyme activity of ADH III was lost rapidly within a few minutes [110]. A biocatalyst composed of the ALDH-containing membranes of acetic acid bacteria is useful in eliminating off-flavors caused by various middle chain length aliphatic aldehydes that occur in foodstuffs such as wheat flour and soybean meal. When unripe cereal grains or beans are used in flour making or soybean meal production, strong off-flavors cause serious problems. Most aldehydes have a low threshold of off-flavor but once such aldehydes are oxidized to the corresponding carboxylic acids, for which the threshold is quite high, the off-flavors are decreased. Like alcohol yeasts and lactic acid bacteria, acetic acid bacteria are known to be edible microbes and produce no appreciable pathogens. Therefore, there is no problem in using cells of acetic acid bacteria carrying ALDH to improve the quality of foodstuffs. The membrane fraction containing ALDH exclusively produced by deleting ADH III by heating is also a useful enzyme for aldehyde microdetermination [110].

As for NAD(P)-independent formaldehyde dehydrogenase, the enzyme from *Methylococcus capsulatus* Bath purified from the membrane was shown to have PQQ as a cofactor [111]. This is the first report of the purification of NAD(P)-independent formaldehyde dehydrogenase from the membrane fraction of a methylotroph and PQQ-containing formaldehyde dehydrogenase coupled to the electron transport chain via a β-type cytochrome or quinone. The properties of this enzymes showed a number of similarities to the soluble NAD(P)-independent ALDH from *Hyphomicrobium zavarzinii* ZV 580 [112].

Another formaldehyde-oxidizing enzyme is the quinoprotein methanol dehydrogenase from methylotrophic and methanotrophic bacteria [2, 113]. So far, formaldehyde dehydrogenase, including both NAD(P)-dependent and NAD(P)-independent enzymes, has been studied mainly in bacteria and yeasts that are able to grow on C1 compounds such as methane and methanol as sole carbon source. It is interesting to see that formaldehyde oxidizing ADH III has recently been found in *Acetobacter* sp. [114].
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