ELECTROMICROBIAL AND RELATED APPROACHES TO ANAEROBIC BIOTRANSFORMATIONS

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Introduction and Overview

There is now a substantial interest in the exploitation of anaerobic microorganisms for the production of fine chemicals by the biotransformation of xenobiotics (e.g. Zeikus 1980, 1983, Morris 1983, Simon & Günther 1983, Simon et al 1985a, Yamada & Shimizu 1988, Morris 1989). Especially because of their ability to maintain a low redox potential in vivo (Jacob 1970, Kjaergaard 1977), due to their possession of a great many low-potential electron carriers, work with such organisms has concentrated upon bioreductions. As recently reviewed (Wong and Drueckhammer 1985, Lovitt et al 1987, Pugh et al 1988; Japanese 1988 book), another area where these organisms have assumed prominence is in the recycling of reduced pyridine nucleotides.

Many of these studies have relied upon the fermentative metabolism of these cells to produce the necessary reducing power. However, such an approach has several disadvantages: (i) there is little control over the reactions taking place, (ii) the desired end-product must be purified from the products of fermentative metabolism, (iii) the available redox potential attainable is limited by the thermodynamics of the reactions of fermentative metabolism itself. Notwithstanding, certain redox couples, for instance H_2/H^+, CO/CO_2 and HCOOH/HCHO, possess generally acceptably low midpoint potentials (Fig 1), and create or consume gaseous or otherwise non-toxic and easily-removed products, and recent attention in this area has indeed concentrated on these (White et al 1987).

A more convenient approach would be simply to generate the necessary reducing power at an electrode. Whilst several purified proteins have been shown, under appropriate conditions, to be more-or-less reveribly electroactive (Armstrong et al 1986, Frew & Hill 1988), and may usefully be exploited for biotransformations (Hill et al 1985), intact cells are for practical faradaic purposes electroinactive. Thus to effect the transfer of reducing power from an electrode to the interior of a cell, it is necessary to add one or more redox mediators of relatively low molecular weight (Fultz & Durst 1982). The

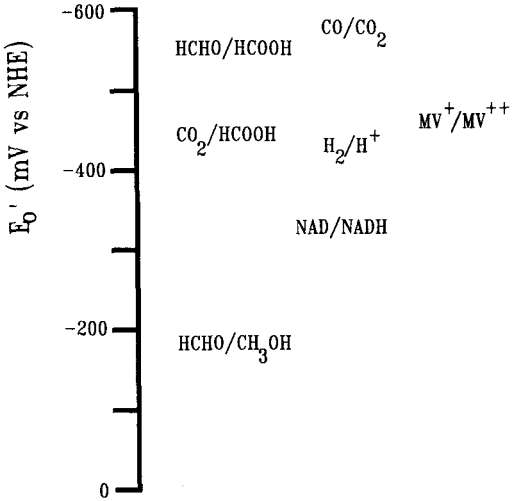

Fig 1. The redox potential of some couples of significance in anaerobic bioreductions. Data from references cited in the text and in Kell et al (1981).

properties required of such mediators, both for this purposes and indeed for use in biofuel cells (Bennetto 1984) and in amperometric biosensors (Turner et al 1987) include: (i) reversible electrochemistry at the cathode of interest, with a well-defined n value, (ii) the ability rapidly and reversibly to penetrate the cell envelope of the organism of interest, (iii) the possession of a mid-point potential suitable for the purpose intended, (iv) a lack of cellular toxicity, (v) preferably an electrical charge different from the product of the biotransformation (permitting an easy work-up by ion-exchange chromatography), (vi) easy availability at a low cost. An additional benefit of the electromicrobial approach is that the rate of the reaction of interest, when limited by the cellular reductase activity, may be measured in real time, simply as the rate of faradaic current flow.

Simon and colleagues (Simon et al 1985a,b, 1986, 1987) have shown that for chemotrophic anaerobes, the viologen dyes (Bird & Kuhn 1981) appear to be ideal redox mediators when Hg is used as the cathode; in addition, not only do viologens such as the methyl viologen (1,1'-dimethyl,-4,4'-bipyridilium) cation serve as suitable mediators at low redox potentials (<-300 mV vs SHE), but many chemotrophic anaerobes contain an exceptionally high methyl viologen-NAD reductase activity (Simon et al 1985a,b, 1986, James et al 1988). It is also worth remarking that Hg when

used as a cathode, and in contrast to the Hg^{++} cation, is non-toxic to microorganisms.

Reductions of the greatest present interest include: R-COOH --> R-CHO, RCHO --> RCH_2OH and R(C=O)R'--> RCH(OH)R' (in 2 chiral forms). Further, the addition of a methylene group to a carboxylate (i.e. RCOOH + CO_2 --> RCH_2COOH), as carried out by a variety of anaerobic microorganisms, is a reductive carboxylation. In what follows, we shall give examples of each of these.

Electromicrobial techniques of use in the study and improvement of anaerobic (and indeed aerobic) biotransformations are not confined to faradaic reactions. We may here make mention of the increasingly important electroporation technique (Potter 1988), for the reversible or irreversible permeabilisation of cells for the introduction of membrane-impermeant molecules, including DNA. Finally, non-faradaic measurements ("dielectric spectroscopy") permit the real-time estimation of cellular biomass (Harris et al, 1987) and thus a rapid assessment of the toxicity of xenobiotics which may be added as substrates or solvents during biotransformations (Stoicheva et al, 1989).

Faradaic methods in anaerobic biotransformations.

As described in full elsewhere (Lovitt et al 1987, James et al 1988, Dixon et al 1989), the strategy used is to prereduce MV in an Hg pool electrode and if appropriate add it to an analytical DME, add the cells of interest, monitor any background current (due typically to hydrogenase) and then add the oxidised xenobiotic of interest, Successful electromicrobial reduction is then manifested as an increased current. A typical faradaic reaction leading to the reduction of a xenobiotic is shown in Fig 2. Here (EWJ et al, unpublished observations), cells of Clostridium La1 (Thanos & Simon 1987) were used as the biocatalyst for the reduction of the chiral beta-ketoester (Christen & Crout 1988) p-Cl-Ph-S-$COCH_2$-$COOCH_3$. The pH-dependence (Fig 3) of the reduction shows an optimum at approximately 6, where the rate of reaction (*ca 200 nmol.(min.mg dw)$^{-1}$*) is very respectable. The chirality of the product has not yet been determined.

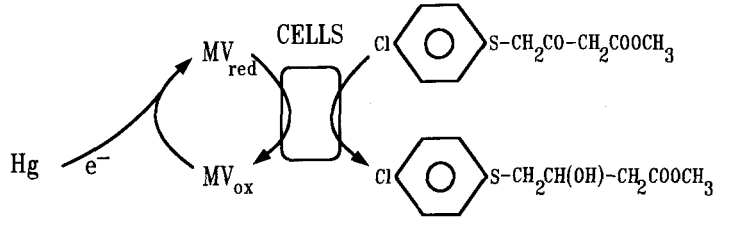

Fig 2. Reduction of a beta-ketoester using an Hg electrode, MV and cells of Clostridium La1.

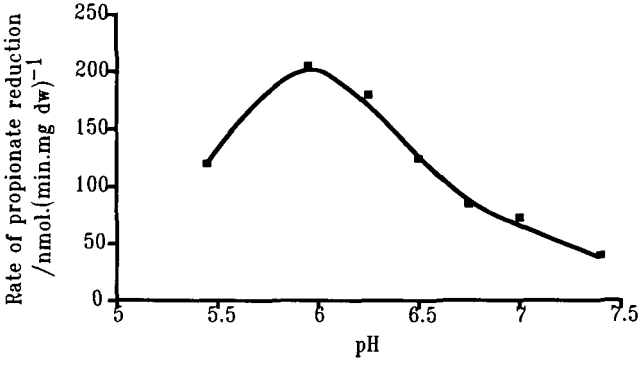

Fig 3. pH-dependence of the rate of the reaction shown in Fig 2.

We and Simon and colleagues (opera cit.) have applied this general approach (mainly with permeabilised cells) using a variety of anaerobic reductases, and suffice to say that one may ring all the expected changes with organisms (and their mode and phase of growth), substrates and environmental conditions such as pH and temperature. Rates as high as 2 umol.(min.mg dw)$^{-1}$ have been obtained for the reduction of NAD^+ using Cl. sporogenes (James et al 1988).

Another interesting reaction is that of reductive carboxylation. For instance, Tyssee (1976) describes the purely electrochemical carboxylation at an Hg electrode of substituted acetonitriles according to the reaction RR'C(CN)H + CO_2 --> RR'C(CN)COOH. We have studied the reaction RCOOH + CO_2 --> RCH_2COOH using the electromicrobial approach (James et al 1988, Dixon et al 1989), with acetyl phosphate as the initial substrate, also in Cl. sporogenes. Because this organism contains pyruvate carboxylase, pyruvate formed may further be converted to give oxaloacetate (and, if NH_4^+ is present, even alanine and aspartate) (Dixon et al 1989). Thus it is possible to build C_4 compounds from C_2 compounds, CO_2 and electrons!

The reduction of carboxylates to alcohols is normally thermodynamically unfavourable, because of the very low mid-point potential (-550 mV) of the RCHO/RCOOH couple (Fig 1), and thus can not be driven by, say, reduced pyridine nucleotides (E_o' = -320 mV) or hydrogen (E_o' = -420 mV). However, recognising that the CO/CO_2 couple has an E_o' of some -560 mV, Simon et al (1987) and White et al (1987) incubated resting cells of Cl. thermoaceticum with CO and the carboxylate of interest, and found that when MV was present the alcohol was indeed formed with, in appropriate cases, some stereoselectivity. This reaction obviously lends itself to the electromicrobial approach, but since the CO produces the non-toxic CO_2, the approach has not

been pursued in any detail. Fraisse and Simon (1988) found similar activities in the related Cl. formicoaceticum (albeit with a different substrate range), and showed that formate was another convenient electron donor. Most recently, White et al (1989) purified the first enzyme of the "carboxylate reductase", and found it to be a tungstoenzyme which could catalyse the further reduction of the aldehydes formed. The great advantages of these organisms, which are rather atypical clostridia in that they possess electron transport chains containing menaquinone and cytochromes (Gottwald et al 1975), is that the carboxylates are reduced in a non-activated form and not, for instance, as CoA-derivatives.

Although the rates of reaction and the substrate range are not as great as one would wish, the success of the work so far encourages one to study the system further, and we have carried out a detailed physiological study of both clostridia in chemostat culture (in preparation). The results of this study lend some (limited) support to the view that the normal function of this reaction in growing cells, as in many gratuitous reductions of xenobiotics, is simply as an electron sink. However, the differences between the two organisms (e.g. an opposite dependence of carboxylate reductase activity on dilution rate in carbon-limited chemostats) suggest that more subtle (if teleological) reasoning should be invoked. That this type of reaction may be far more widespread than has hitherto been supposed is instanced by its indirect observation in lactobacilli, which cause off-flavours of wines containing sorbic acid (2,4-hexadienoic acid) by, inter alia, its reduction to 2,4-hexadien-1-ol followed by a variety of isomerisations and esterifications (Crowell & Guymon 1975).

A remarkable characteristic of anaerobic bacteria is their ability to use as terminal electron acceptors compounds that one does not normally even consider as redox-active (Thauer & Morris 1984, Schink 1986, Berry et al 1987). In view of the success of reductions exploiting the CO/CO_2 couple in which the electron donor reaction is $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$, it seems plausible that a similar reaction involving nitrogen oxides might be expected to occur in nature, i.e. the anaerobic growth of bacteria on NO. We do not know the mid-point potential of the NO/NO_2 couple when participating in the analogous reaction, but this seems an avenue well worth exploring.

Other electromicrobial techniques

In recent years, there has been much interest in the application of relatively high electric field pulses to cells, to induce electroporation (for the uptake of foreign, membrane-impermeant substances) and even electrofusion (Zimmermann 1982). We have studied ("irreversible") electroporation as a means of rendering cells permeable to water-soluble, membrane-impermeant substrates suitable for biotransformation, and have found it very suitable. As an interesting sideline, we report the experiment displayed in Fig 4, in which it may be observed that the application (using a BTX Transfector 100 instrument) of a field of 1 kV/cm for 3.75 ms to a suspension of Bacillus megaterium results in an apparent increase in cell number. Since this

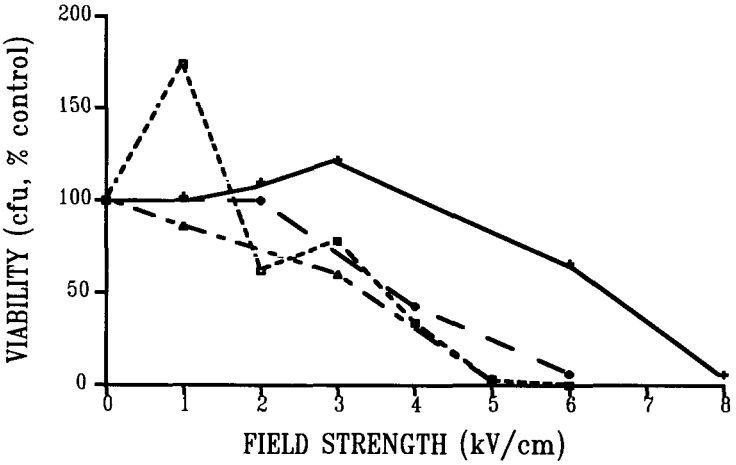

Fig 4. Irreversible electroporation (as monitored by viability) of bacteria. Cells were exposed to a 5 ms pulse of the field strength indicated in a BTX Transfector 100, and plated out on appropriate agar plates. Cells sued were Escherichia coli JMW7 (_____), Bacillus megaterium GW1 (.....), Clostridium acetobutylicum NCIB 8052 (-.-.-.) and Clostridium pasteurianum ATCC 6013-MR505 (------).

species has a tendency to grow in filaments, it is assumed that the field causes **electrofission** of these filaments so as to increase the potential number of colony-forming units. Thus we may add electrofission to the possible effects induced by high electrical fields on intact microbial cells. This experiment also shows that Gram-negative bacteria are somewhat more resistant than are Gram-positive bacteria to irreversible electroporation.

Concluding remarks

In this very brief overview, we hope to have been able to show that the application of electromicrobial techniques to the study of anaerobic biotransformations has thrown up a great many most interesting features, some of which have every possibility of leading to processes of commercial interest.

Acknowledgments

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