Direct electrochemical studies of hydrogenase and CO dehydrogenase

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The reduction potentials of two relatively high-molecular-mass enzymes were determined directly at an edge pyrolytic graphite electrode by using square-wave voltammetry. The equilibrium reduction potential *versus* standard hydrogen electrode was determined for *Clostridium pasteurianum* hydrogenase I ($E'_0 = -377 \pm 10$ mV; molecular mass 60 kDa) and *Rhodospirillum rubrum* carbon monoxide dehydrogenase ($E'_0 = -418 \pm 7$ mV; molecular mass 62 kDa). The reduction potential of each enzyme was pH-independent, and one electron was transferred per redox centre. The reduction potential was determined to be identical for the CO dehydrogenase, semi-apo-(CO dehydrogenase), and CO dehydrogenase with carbonyl sulphide (COS) or cyanide bound. The electron-transferring efficiency of CO dehydrogenase was affected by two inhibitors, COS and cyanide, as indicated by a diminished analytic current.

INTRODUCTION

In the last decade, developments in unmediated bioelectrochemistry have permitted direct determination of reduction potentials for several low-molecular-mass-electron-transfer proteins at solid electrodes (Armstrong *et al.*, 1987, 1989; Frew & Hill, 1988; Hagen, 1989). Here we report for the first time direct electrochemistry for two *enzymes* of relatively high molecular mass which contain iron-sulphur redox centres.

The oxidation and reduction of substrates in bacterial cell respiration is often catalysed by iron-sulphur enzymes, including hydrogenase and carbon monoxide dehydrogenase (CODH). In the fermentative bacterium *Clostridium pasteurianum*, protons serve as one of the terminal electron acceptors in metabolism. Hydrogenase I, a monomer with a molecular mass of 60 kDa, catalyses the reversible reduction of protons to molecular hydrogen in the presence of suitable electron donors as follows:

Scheme 1

The optimal activity of hydrogenase I has been shown to be pH-dependent (Adams & Mortenson, 1984). From e.p.r. spectroscopy, four [4Fe-4S] clusters and a novel 'hydrogen' iron-sulphur cluster have been identified, and at pH 8 both types of redox centres had a reduction potential of -420 mV (Adams, 1987).

In the photosynthetic bacterium *Rhodospirillum rubrum*, CO dehydrogenase is specifically induced by CO and catalyses the oxidation of CO to CO_2 in the presence of suitable electron acceptors as follows (Bonam & Ludden, 1987):

Scheme 2

CODH, a monomer with a molecular mass of 62 kDa, appeared to contain two [4Fe-4S] clusters and one atom of nickel (Hyman *et al.*, 1989). It was shown (Ensign *et al.*, 1989a) that CODH had a pH-dependent activity and reversibly ligated the CO analogues, cyanide and carbonyl sulphide (COS). Semi-apo-CODH, which contained only two [4Fe-4S] clusters, was reversibly activated

with nickel (Bonam & Ludden, 1987) and did not ligate cyanide (Ensign *et al.*, 1989b). Since semi-apo-CODH had little enzymic activity and did not ligate the CO analogue, it was concluded that nickel was at the active site (Ensign *et al.*, 1989b).

Since both hydrogenase I and CODH catalyse reactions involving protons and both their activities are pH-dependent, it was of interest to determine how the pH effects their equilibrium redox properties, especially since there is a fundamental difference between a redox process that requires protonation of different oxidation states of the enzyme and one that requires the protonation of different oxidation states of substrate. We have previously used square-wave voltammetry (s.w.v.), a rapid and sensitive electrochemical technique, to identify both pH-dependent and pH-independent reduction potentials of bacterial ferredoxins (Smith & Feinberg, 1990).

The relatively uncomplicated [4Fe-4S] clusters found in ferredoxins may serve as models for the redox centres found in iron-sulphur enzymes. Many iron-sulphur enzymes, including nitrogenase and hydrogenase, catalyse redox reactions involving the transfer of multiple electrons, typically in single electron-transfer steps. For example, both kinetic studies (Van Dijk *et al.*, 1979) and equilibrium redox titrations (Adams, 1987) support a mechanism for hydrogenase that involves multiple single electron-transfer steps. Multiple [4Fe-4S] clusters within the enzyme, each of which transfers a single electron, are believed to exchange electrons between the enzyme active site and other redox molecules (Adams, 1987). S.w.v. can be used to determine unambiguously the number of electrons transferred per redox centre. We also compared the spectroscopic properties of *C. thermosaccharolyticum* ferredoxin, which has a known pH-dependent reduction potential (Smith & Feinberg, 1990), with those of CODH.

EXPERIMENTAL

Protein sources and electrolytes

The ferredoxin from *C. thermosaccharolyticum* (A.T.C.C. 7956) was purified, and concentrations were determined by procedures previously described (Smith & Feinberg, 1990). The

Abbreviations used: CODH, carbon monoxide dehydrogenase; Fd, ferredoxin; HiPIP, high potential iron-sulphur protein; COS, carbonyl sulphide; $[\text{Cr}(\text{en})_3]\text{Cl}_3$, tris-(1,2-diaminoethane)chromium(III) salt; SHE, standard hydrogen electrode; s.w.v., square-wave voltammetry; ox., oxidized; red., reduced.

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hydrogenase I from *C. pasteurianum* (A.T.C.C. 6013) was partially purified by procedures previously described (Nakos & Mortenson, 1971) and further purified anaerobically by means of additional anion-exchange chromatography. The enzyme concentration was determined at 420 nm from the molar absorptivity of $24400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Adams & Mortenson, 1984). *R. rubrum* CODH, semi-apo-CODH and CODH bound with COS or cyanide were prepared, concentrations were determined and activities were assayed as previously described (Ensign *et al.*, 1989a,b).

NaCl, MgCl_2 , and BaCl_2 were purchased from Sigma, Mallinckrodt, and EM Science (Cherry Hill, NJ, U.S.A.) respectively. Tris-(1,2-diaminoethane)chromium(III) salt $\{[\text{Cr}(\text{en})_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}\}$ was synthesized according to the procedure developed by Gillard & Mitchell (1972).

Concentrated enzyme solutions (hydrogenase I in 25 mM-Tris/HCl, pH 8, and CODH in 25 mM-Mops, pH 7.5) were diluted or dialysed anaerobically into the appropriate buffer (potassium phosphate, pH 6.4–7.5; Tris/HCl, pH 8.0–8.7) and the various supporting electrolytes. The specific concentrations of electrolyte $\{\text{MgCl}_2$, BaCl_2 , NaCl, or $[\text{Cr}(\text{en})_3]\text{Cl}_3\}$ used to maximize protein-electrode interactions were based on a previous protocol (Armstrong *et al.*, 1987).

Spectroscopic methods

Concentrated protein solutions in 10 mM-Tris/HCl, pH 8, were anaerobically diluted at least 20-fold into the appropriate buffer. All spectra were recorded anaerobically under an H_2/N_2 (1:19) atmosphere. U.v.-visible spectra were recorded on a computer-interfaced Cary 219 spectrophotometer using stoppered quartz cuvettes of 1 cm path length. C.d. spectra were recorded on a Jasco J-500A spectrapolarimeter at a scan rate of 50 nm/min and a resolution of 0.4 nm using a stoppered circular quartz cell with a path length of 1 cm. The c.d. spectra were signal-averaged twice, corrected for background, and normalized for concentration. Difference-c.d. spectra were smoothed twice using a floating 9-point least-squares regression analysis (Savitzky & Golay, 1964).

Reduction potentials

All electrochemical experiments were performed using a standard three-electrode configuration as previously described (Smith *et al.*, 1991; Hagen, 1989). The working electrode was edge-polished pyrolytic graphite, the counter-electrode was a platinum wire sealed in borosilicate and the reference was a saturated Ag/AgCl electrode. The reduction potential of the saturated Ag/AgCl reference ($E'_0 = 199 \text{ mV}$) was verified against a saturated calomel electrode ($E'_0 = 244 \text{ mV}$) (Sawyer & Roberts, 1975). The electrochemical cell was made anaerobic by cycling between a vacuum and purified argon passed over a heated R3-11 catalyst (Chemical Dynamics Corp., South Plainfield, NJ, U.S.A.). An anaerobic 25–50 μl sample containing 30–150 μM -protein in the supporting electrolyte and appropriate buffer was placed on the inverted working electrode via a Hamilton gas-tight syringe.

All current/potential data from the s.w.v. experiments were recorded using either a BAS-100 or computer-interfaced PARC 273 potentiostat, as previously described (Smith & Feinberg, 1990). Equilibrium reduction potentials were determined from the voltammograms at the applied potential at which the peak current occurred. The number of electrons transferred per redox centre was determined from the peak width at half height ($W_{1/2} = 126 \text{ mV}/n$) (n is the number of electrons transferred/redox site per molecule). All potentials in this paper are against a standard hydrogen electrode (SHE).

THEORY

As discussed in the Introduction, there is a fundamental difference between a redox process that requires protonation of different oxidation states of the enzyme, and one that requires the protonation of different oxidation states of substrate. Scheme 3, which follows, is one way to represent the two half-reactions and overall reaction for the enzymic catalysis of a proton-linked reduction of substrate:

Scheme 3

where E and S are enzyme and substrate respectively. In this Scheme, since the enzymic half-reaction does not involve proton exchange, its reduction potential is not pH-dependent. The following Nernst equation (in mV), based on Scheme 3, can be written:

$$E_{\text{ambient}} = (E_{\text{ox.}} - E_{\text{os}}) + 59 \log \frac{[\text{E}_{\text{ox.}}][\text{S}^-]}{[\text{E}_{\text{red.}}^-][\text{H}^+ \cdot \text{S}]} + 59 \log [\text{H}^+] \quad (1)$$

where E_{ambient} is the ambient potential, $E_{\text{ox.}}$ and E_{os} are the equilibrium reduction potentials of the enzyme and substrate respectively. Both electrochemical and spectroscopic methods can be used to test the validity of Scheme 3 as expressed in eqn. (1). For example, the ambient potential (E_{ambient}) at a specific pH can be measured potentiometrically for the bulk solution at a Methyl Viologen-modified gold electrode (Smith & Feinberg, 1990) as the indicating electrode. The relative oxidation states of enzyme ($[\text{E}_{\text{ox.}}]/[\text{E}_{\text{red.}}^-]$) at a specific pH can be measured by u.v.-visible spectroscopy.

S.w.v. can also be used to test the validity of eqn. (1). A potential is applied to the working electrode to establish an ambient potential at the electrode surface, and the change in concentrations of oxidized and reduced species is directly measured in the form of current. The equilibrium concentrations of oxidized and reduced species at the electrode surface is governed by the Nernst equation. For a reversible redox couple, the observed net current reaches a maximum where $[\text{E}_{\text{ox.}}] = [\text{E}_{\text{red.}}^-]$. $E_{\text{ox.}}$ is determined from the applied potential at which the maximum net current, i_p , is observed. If electron transfer of the enzyme is proton-linked (i.e. $\text{E}_{\text{ox.}} + \text{e}^- + \text{H}^+ \rightarrow \text{HE}_{\text{red.}}$), a 59 mV/ n shift in reduction potential per pH unit would be observed.

RESULTS

Hydrogenase

It was determined from the experimental voltammograms that the reduction potential for hydrogenase I was -377 mV at both pH 6.4 and pH 8.0, and that, at both pH values, one electron was transferred per redox site (see Fig. 1). The visible spectra of *C. pasteurianum* hydrogenase I at pH 6.4 and pH 8.6 is shown in Fig. 2. E_{ambient} was measured potentiometrically to be -353 mV and -460 mV at pH 6.4 and pH 8.0 respectively.

CO dehydrogenase

The c.d. spectra of *R. rubrum* CODH at pH 6.4 and pH 8.7 are shown in Fig. 3. The difference-c.d. spectra of *R. rubrum* CODH and *C. thermosaccharolyticum* ferredoxin (Fd) at pH 6.4 and pH 8.7 is shown in Fig. 4. The difference in the ellipticity values of CODH at the two different pH values, in contrast with those of *C. thermosaccharolyticum* Fd, indicate that the electronic transitions of the iron-sulphur centres are pH-independent.

Fig. 1. S.w.v. of 30 μM *C. pasteurianum* hydrogenase I in 0.1 M-NaCl/50 mM-phosphate, pH 6.4

The step potential was 1 mV, the pulse potential was 50 mV and the frequency of the applied potential pulse was 2 Hz.

Fig. 2. U.v.-visible spectra of 7 μM *C. pasteurianum* hydrogenase I in 50 mM-phosphate, pH 6.4 (—) and 50 mM-Tris/HCl, pH 8.4 (---)

The increased absorbance at 400 nm at pH 6.4 is indicative of an increased concentration of oxidized iron-sulphur clusters.

Fig. 3. C.d. spectra of 20 μM *R. rubrum* CO dehydrogenase in (a) 25 mM-NaCl/25 mM-phosphate, pH 6.4 and (b) 25 mM-NaCl/25 mM-Tris/HCl, pH 8.7

The spectra are offset 1 degree/ $\text{M}\cdot\text{cm}$. $\Delta\epsilon$ is the molar c.d. absorption coefficient.

The direct electrochemical results for CODH are summarized in Table 1. It was determined from the voltammograms (see Fig. 5) that the reduction potential for native CODH was pH-independent, and that one electron was transferred per redox

Fig. 4. Difference c.d. spectra pH 6.4 and pH 8.7 for (a) 7 μM *C. thermosaccharolyticum* Fd and (b) 20 μM *R. rubrum* CODH

The difference spectra are offset 1 degree/ $\text{M}\cdot\text{cm}$. $\Delta\epsilon$ and its units are given in Fig. 3.

Table 1. S.w.v. response for CODH

Sample	Conditions	E'_0 (mV)	W_i (mV)	i_p ($\mu\text{A}/\mu\text{M}$)*
Apo-CODH	0.1 mM-Cr(en) ₃ Cl ₃ , pH 7.5	-416	125	3.8
	10 mM-NaCl, pH 7.5	-420	130	3.9
CODH	25 mM-BaCl ₂ , pH 7.5	-408	125	1.2
	50 mM-MgCl ₂ , pH 7.5	-410	125	1.1
	0.1 mM-Cr(en) ₃ , pH 7.5	-416	130	0.9
	10 mM-NaCl, pH 7.5	-423	130	0.8
	10 mM-NaCl, pH 6.4	-422	130	0.9
	10 mM-NaCl, pH 8.7	-428	135	0.8

* Normalized for concentration.

site. The reduction potential for both native and semi-apo-CODH was determined to be -418 ± 7 mV. However, the reduction potentials for CODH bound to the inhibitors, COS and cyanide, do not appear to be significantly different from native and semi-apo-CODH. Fig. 5(c) shows the voltammogram of cyanide bound to CODH, which is very similar to that observed for COS bound CODH (result not shown).

The magnitude of the peak current normalized for concentration was typically four times greater for semi-apo-CODH than for native CODH, as shown in Table 1. The voltammograms for native, semi-apo, and cyanide forms of CODH are shown in Fig. 5. The forms of CODH with COS or cyanide bound had very similar voltammograms, and their reduction potentials did not appear to be significantly different from those of either native or semi-apo-CODH. The inhibitors, COS and cyanide, both significantly reduced the peak current (Fig. 5c). The concentrations and type of supporting electrolyte {BaCl₂, MgCl₂, NaCl or

Fig. 5. S.w.v. of (A) 0.11 mM *R. rubrum* semi-apo-CODH in 0.1 M-NaCl, (B) 0.11 mM native *R. rubrum* CODH in 0.1 M-NaCl and (C) 0.08 mM *R. rubrum* CODH ligated to cyanide in 50 mM-NaCl/20 mM-BaCl₂.

The buffer was 50 mM-Mops, pH 7.5. The step potential was 1 mV, the pulse potential was 50 mV and the frequency of the applied potential pulse was 2 Hz.

[Cr(en)₃]Cl₃ also did not significantly effect peak position or shape. Additional background current, the source of which is not known, was observed in voltammograms of CODH bound to either COS or cyanide.

DISCUSSION

We determined the reduction potential for both hydrogenase I and CODH to be about -400 mV, and one electron was transferred per redox site, which is characteristic of low-potential [4Fe-4S] clusters. As would be expected, the reduction potential of each enzyme is near the potential of the reaction it catalyses. For example, the reduction potential of hydrogenase I was determined to be -377 mV, and the reduction potential of the 2H⁺/H₂ redox couple is -413 mV at pH 7. Thus the oxidation of molecular hydrogen by hydrogenase I is thermodynamically more favourable at physiological pH. The reduction potential of CODH was determined to be -418 mV, and the reduction potential of the CO₂/CO redox couple is -518 mV at pH 7. Notably, the reduction potential of CODH is 103 mV more positive than the midpoint potential of the reaction it catalyses, which is reasonable, since CODH *only* catalyses CO oxidation.

Hydrogenase

It was determined from the voltammograms at different pH values that the reduction potential of hydrogenase I is pH-independent. Since the hydrogenase I half-reaction does not involve an obligatory proton exchange, as presented in Scheme 3, the reduction potential need not be pH-dependent. The reduction potential at both pH 6.4 and pH 8.0 obtained in this work by s.w.v. ($E'_0 = -377 \pm 10$ mV) is in reasonable agreement with the value obtained through e.p.r. redox titrations at pH 8.0 [$E'_0 = -420 \pm 30$ mV (Adams, 1987)].

Both the oxidation state of hydrogenase I and E_{ambient} were found to be pH-dependent as predicted by eqn. (1), which is based on Scheme 3. It is important to note that many alternative schemes, including schemes that have a pH-dependent electron-

transfer step for enzyme, can be used to describe an overall equilibrium with net proton exchange. For example, five different expressions of the Nernst equation have been used to calculate different redox properties and reduction potential of *C. pasteurianum* Fd (Sobel & Lovenberg, 1966; Tagawa & Arnon, 1968; Eisenstein & Wang, 1969; Packer & Sternlicht, 1975; Magliozzo *et al.*, 1982). Nonetheless, our voltammetric, potentiometric and spectroscopic results are all in agreement with Scheme 3, which suggests that the reduction potential of hydrogenase I is pH-independent.

CODH

Surprisingly, despite its lower enzymic activity, semi-apo-CODH appears to transfer electrons at a higher rate than does holo-CODH, as determined from the larger normalized peak currents shown in Table 1. Both the cyanide- and COS-bound holoenzyme, both of which also have low enzymic activities, had the smallest normalized peak currents (see Fig. 5 for the voltammogram of cyanide-bound CODH). Electron-transfer rates, in contrast with the reduction potential, depend on the nature of the active site in CODH. The mechanism for electron transfer is not obvious from the voltammetric results.

Although CODH contains nearly 20 histidine residues (Bonam & Ludden, 1987), the c.d. spectra (Fig. 4) and reduction potential of CODH are unaffected by changes in pH. We attribute the differences in ellipticity values for *C. thermosaccharolyticum* Fd, which is even greater than those previously reported for high-potential iron-sulphur proteins (HiPIPs) that contain histidine-49 (Przysiecki *et al.*, 1985), to the different ionization states of histidine-2, which is located near iron-sulphur cluster II. Similarly, an ionizable histidine residue located near a [4Fe-4S] cluster was concluded to be the reason for the pH-dependent reduction potential of both *C. thermosaccharolyticum* Fd (Smith & Feinberg, 1990) and HiPIPs containing histidine-49 (Przysiecki *et al.*, 1985).

Redox potential of different redox centres

In this and previous studies (Adams, 1987), no difference in reduction potential was observed between the [4Fe-4S] and 'hydrogen' clusters of hydrogenase I. Similarly, in the present study, no difference in reduction potential was observed between the two [4Fe-4S] clusters and nickel in CODH. For that matter, it is not established whether or not nickel in CODH even undergoes a change in oxidation state during catalysis.

For both enzymes it is possible that only one [4Fe-4S] cluster equilibrates with the electrode and then exchanges electrons to other redox centres within the protein. S.w.v. would not be able to detect any difference between the reduction potential of individual redox centres if (a) the rate of electron transfer between a [4Fe-4S] centre and the electrode was faster than intramolecular electron transfer between individual redox centres or (b) the reduction potential of the individual redox centres were isopotential. In general, it is difficult to distinguish small differences in the reduction potentials of different redox centres within the same protein with either spectroscopic or electrochemical techniques. However, differences as small as 40 mV between different redox centres in cytochrome *c*₃ were reported by curve-fitting data obtained from an analogous electrochemical technique (Bianco & Haladjian, 1981).

Activity versus pH

Both hydrogenase I (Adams & Mortenson, 1984) and CO dehydrogenase (Ensign *et al.*, 1989a) have pH-dependent enzymic activities, suggesting that proton exchange is involved in a rate-limiting step in both their enzymic mechanisms. If electron transfer in these enzymes was proton-linked (i.e. $E_{\text{ox.}} + e^- + H^+ \rightarrow$

HE_{red.}), one would expect the reduction potential of redox centres which transfer one electron ($n = 1$) to shift nearly 140 mV over the pH range examined. Our voltammetric results indicate that the reduction potential of both these enzymes is pH-independent. We therefore conclude that the electron-transfer steps in both their enzymic mechanisms are not proton-linked and not rate-limiting.

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