Ca²⁺ stabilizes the semiquinone radical of pyrroloquinoline quinone

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Spectroelectrochemical studies were performed on the interaction between Ca²⁺ and pyrroloquinoline quinone (PQQ) in soluble glucose dehydrogenase (sGDH) and in the free state by applying a mediated continuous-flow column electrolytic spectroelectrochemical technique. The enzyme forms used were holo-sGDH (the holo-form of sGDH from *Acinetobacter calcoaceticus*) and an incompletely reconstituted form of this, holo-X, in which the PQQ-activating Ca²⁺ is lacking. The spectroelectrochemical and ESR data clearly demonstrated the generation of the semiquinone radical of PQQ in holo-sGDH and in the free state in the presence of Ca²⁺. In contrast, in the absence of Ca²⁺ no semiquinone was observed, either for PQQ in the free state (at pH 7.0) or in the enzyme (holo-X). Incorporation of Ca²⁺ into the active site of holo-X, yielding holo-sGDH, caused not only

stabilization of the semiquinone form of PQQ but also a negative shift (of 26.5 mV) of the two-electron redox potential, indicating that the effect of Ca²⁺ is stronger on the oxidized than on the reduced PQQ. Combining these data with the observations on the kinetic and chemical mechanisms, it was concluded that the strong stimulating effect of Ca²⁺ on the activity of sGDH can be attributed to facilitation of certain kinetic steps, and not to improvement of the thermodynamics of substrate oxidation. The consequences of this conclusion are discussed for the oxidative as well as for the reductive part of the reaction of sGDH.

Key words: calcium ions, pyrroloquinoline quinone, redox potential, semiquinone, soluble glucose dehydrogenase.

INTRODUCTION

Pyrroloquinoline quinone (PQQ) is a cofactor in a number of dehydrogenases occurring in a variety of Gram-negative bacteria. The enzymes concerned are mainly found in the groups of alcohol- and sugar-oxidizing dehydrogenases [1]. All PQQ-containing dehydrogenases require Ca²⁺ for activity, except membrane-bound glucose dehydrogenase (mGDH), which requires Mg²⁺ [2–4]. Although it is clear that these bivalent cations activate PQQ in these enzymes [5–7], the mechanism of this activation is still unclear. One of the possibilities is that activation drastically changes the thermodynamic properties of the cofactor. To investigate this, the effect of Ca²⁺ on the redox potential for the enzyme species which could be involved in the catalytic cycle of soluble glucose dehydrogenase (sGDH) must be known.

Determination of the redox potential of enzymes is problematic [8]. However, recently, the mediated continuous-flow column electrolytic spectroelectrochemical (CFCES) technique, which is based on the spectroscopic detection of the redox state of a protein equilibrated in a continuous-flow redox buffer regulated by column electrolysis, has been developed [9,10]. Since this technique appeared to be successful for the measurement of redox potentials of a variety of redox proteins [9–11], it has been applied in the present study to determine the redox potential of enzyme-bound and free PQQ in the absence and presence of Ca²⁺.

The enzyme used in the present work is sGDH from *Acinetobacter calcoaceticus* [2]. It oxidizes a broad range of aldose sugars to their corresponding lactones, with concomitant reduction of its cofactor PQQ. Structural as well as kinetic evidence has been provided for a mechanism in which direct hydride transfer from the substrate to PQQ is followed by tautomerization of C-5-reduced PQQ to reduced PQQ (PQQ_{red}) as the rate-determining step [12]. Re-oxidation of PQQ_{red} in sGDH occurs readily using

various artificial dyes, some of them acting as one-, others as two-electron acceptors [13–15]. Cytochrome *b*₅₆₂ was found to be the sole cytochrome from this organism that was active as an electron acceptor for sGDH [16]. Fully active sGDH, i.e. the holo-form (holo-sGDH), consists of two identical subunits, two PQQs and six Ca²⁺ ions, four of them involved in the dimerization of the subunits, the other two in activating the two PQQs [7,17]. The PQQ-lacking apo-form of sGDH (apo-sGDH) can be produced in good yield by an *Escherichia coli* recombinant strain [18]. Apo-sGDH can be fully reconstituted to holo-sGDH or incompletely to holo-X, the enzyme form in which the PQQ-activating Ca²⁺ is lacking and which has a very low activity [12]. Thus, to investigate the effect of Ca²⁺ on the thermodynamic properties of PQQ bound in an enzyme, the redox potentials of holo-sGDH as well as of holo-X were determined and the data compared with those for free PQQ in the absence and presence of Ca²⁺ and other cations.

EXPERIMENTAL

Reagents

Phenazine methosulphate (PMS), phenazine ethosulphate (PES) and PQQ were purchased from Wako (Kyoto, Japan), Nacalai Tesque (Kyoto, Japan) and Sigma respectively, and were used without further purification. The inorganic chemicals used were of analytical reagent grade. The standard buffers used in the present work were 30 mM Mops, pH 7.0, and 30 mM Caps, pH 11.0.

Enzyme preparations

Apo-sGDH was isolated from an *E. coli* recombinant strain as described previously [18]. Holo-sGDH was prepared by adding PQQ at an amount equimolar to the subunit in the presence of 3 mM Ca²⁺. Holo-X was prepared in the same way, except that

Abbreviations used: PQQ, pyrroloquinoline quinone (subscripts ox, sem and red denote the oxidized, semiquinone and fully reduced forms of PQQ respectively); sGDH, soluble glucose dehydrogenase; holo-X, artificial form of sGDH lacking PQQ-activating Ca²⁺; mGDH, membrane-bound glucose dehydrogenase; PMS, phenazine methosulphate; PES, phenazine ethosulphate; CFCES, continuous-flow column electrolytic spectroelectrochemistry.

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PQQ was added, in the absence of Ca^{2+} , to apo-sGDH from which adventitious Ca^{2+} was removed [7].

Mediated CFCES

The system used was similar to that described previously [9,10]. In the present work, 10 μl of a sample solution containing approx. 250 μM enzyme form or 500 μM free PQQ was injected on to the mobile phase (30 mM Mops buffer, pH 7.0) in a flow injection analysis mode, and mixed with an electrochemically regulated mediator solution. PMS and/or PES were used as mediator at a final concentration of 50 μM and the mediator solution was prepared immediately before use. The mediator solution and mobile phase buffer were bubbled continuously with argon gas throughout the experiments and the flow-rate was 1.0 $\text{ml} \cdot \text{min}^{-1}$, unless otherwise stated. Further experimental details can be found in previous reports [9,10]. All redox potentials stated in the present study are referred to the standard hydrogen electrode.

Mathematical determination of redox potentials

In considering the reduction of PQQ to PQQ_{red} , a two-step, one-electron transfer (1E1E) mechanism is indicated:

where PQQ_{ox} , PQQ_{sem} and PQQ_{red} denote oxidized, semiquinone and fully reduced forms of PQQ respectively. The Nernst equations of eqn (1) and eqn (2) are given by:

$$\frac{[\text{PQQ}_{\text{ox}}]}{[\text{PQQ}_{\text{sem}}]} = \exp \left[\frac{F}{RT} (E - E_1^{\circ'}) \right] \equiv \eta_1 \quad (3)$$

$$\frac{[\text{PQQ}_{\text{sem}}]}{[\text{PQQ}_{\text{red}}]} = \exp \left[\frac{F}{RT} (E - E_2^{\circ'}) \right] \equiv \eta_2 \quad (4)$$

where E is the electrode potential (or solution potential); $E_1^{\circ'}$ and $E_2^{\circ'}$ are the redox potential of the first and second electron transfer steps respectively. In column electrolytic spectroscopy, the relationship between absorbance (A) and E is expressed by the following equation under equilibrium conditions [19]:

$$A = (\epsilon_{\text{ox}}[\text{PQQ}_{\text{ox}}] + \epsilon_{\text{sem}}[\text{PQQ}_{\text{sem}}] + \epsilon_{\text{red}}[\text{PQQ}_{\text{red}}])l$$

$$= \frac{c_t l}{\eta_1 \eta_2 + \eta_2 + 1} (\epsilon_{\text{ox}} \eta_1 \eta_2 + \epsilon_{\text{sem}} \eta_2 + \epsilon_{\text{red}}) \quad (5)$$

where ϵ_{ox} , ϵ_{sem} and ϵ_{red} are the absorption coefficients of the corresponding species, c_t ($= [\text{PQQ}_{\text{ox}}] + [\text{PQQ}_{\text{sem}}] + [\text{PQQ}_{\text{red}}]$) denotes the total concentration of quinonoid compounds, l is the light-path length. Since $\epsilon_{\text{ox}} c_t l$ and $\epsilon_{\text{red}} c_t l$ can be evaluated experimentally, A versus E curves can be analysed, based on eqn (5), using $E_1^{\circ'}$, $E_2^{\circ'}$ and ϵ_{sem} as adjustable parameters, by means of a non-linear least-squares method.

The difference between $E_1^{\circ'}$ and $E_2^{\circ'}$ reflects the thermodynamic stability of the semiquinone intermediate, as expressed by the semiquinone formation constant (K_{sem}):

$$K_{\text{sem}} \equiv \frac{[\text{PQQ}_{\text{sem}}]^2}{[\text{PQQ}_{\text{ox}}][\text{PQQ}_{\text{red}}]} = \exp \left[\frac{F}{RT} (E_1^{\circ'} - E_2^{\circ'}) \right] = \frac{\eta_2}{\eta_1} \quad (6)$$

where K_{sem} is formally the equilibrium constant of the comproportionation reaction: $\text{PQQ}_{\text{ox}} + \text{PQQ}_{\text{red}} \leftrightarrow 2\text{PQQ}_{\text{sem}}$, although this would not be an elementary reaction in enzyme-bound PQQ.

If K_{sem} is significantly smaller than unity ($E_1^{\circ'} - E_2^{\circ'} > 100$ mV), the electron transfer of quinonoid compounds can be considered to be a one-step, two-electron transfer (2E) mechanism given by:

Analysis of the A versus E curves in this case can be performed, based on eqn (8), for the determination of the overall two-electron redox potential ($E^{\circ'} [= (E_1^{\circ'} + E_2^{\circ'})/2]$):

$$A = (\epsilon_{\text{ox}}[\text{PQQ}_{\text{ox}}] + \epsilon_{\text{red}}[\text{PQQ}_{\text{red}}])l$$

$$= \frac{c_t l}{\eta + 1} (\epsilon_{\text{ox}} \eta + \epsilon_{\text{red}}) \quad (8)$$

with

$$\eta \equiv \frac{[\text{PQQ}_{\text{ox}}]}{[\text{PQQ}_{\text{red}}]} = \exp \left[\frac{2F}{RT} (E - E^{\circ'}) \right] \quad (9)$$

Spectroscopy

UV-visible absorption spectra were recorded using a MultiSpec-1500 photodiode-array spectrophotometer (Shimadzu). ESR spectra were recorded on an ES-10 spectrometer with a glass capillary cell with an inner diameter of 0.7 mm (Nikkiso). The magnitude of the modulation (100 kHz) was selected to be as low as possible to optimize the resolution and signal-to-noise ratio of observed spectra. The microwave power was set to 1–2 mW. All measurements were performed at room temperature.

RESULTS

Redox characteristics of enzyme-bound PQQ

Although holo-sGDH did not show an electrochemical signal at conventional electrodes (results not shown), it exhibited reversible redox behaviour with CFCES using PES as a mediator ($E_{\text{PES}}^{\circ'} = 55$ mV at pH 7.0). The spectral changes occurring during the potential change are shown in Figure 1. The spectra were almost independent of the flow rate up to 2.0 $\text{ml} \cdot \text{min}^{-1}$. These results strongly support the idea that a redox equilibrium was attained between holo-sGDH and PES in the column. Figure 1 inset shows the E dependence of A_{338} and A_{485} , indicating the generation of PQQ_{sem} in holo-sGDH at approx. $E = 10$ mV. The reduced form of holo-sGDH has a maximum at A_{338} , and the small absorption band at approx. A_{480} is characteristic of PQQ_{sem} . These observations are very similar to those found for PQQ-methanol dehydrogenase [20]. Therefore A versus E plots in the inset of Figure 1 were analysed based on eqn (5) for the 1E1E mechanism. As is clear from the solid lines, the experimental data fitted well into this equation and the calculated values of $E_1^{\circ'}$ and $E_2^{\circ'}$ were 33 mV and -12 mV respectively ($E^{\circ'} = 10.5$ mV, $K_{\text{sem}} = 5.8$). The 2E mechanism was not suitable for reproduction of the data, even for the sigmoidal plots at A_{338} , as shown by the broken line in the inset of Figure 1.

Formation of PQQ_{sem} in holo-sGDH also occurred when holo-sGDH was reduced by a sub-stoichiometric amount of glucose under anaerobic conditions with subsequent exposure of the reduced holo-sGDH solution to air. The spectral changes observed during the auto-oxidation process are shown in Figure 2, the absorbance at 480 nm in spectrum B was indicative of the generation of PQQ_{sem} . This was confirmed by the detection of an ESR signal under these conditions, as shown in the inset of Figure 2, which was also similar to that of methanol dehydrogenase [21]. This is the first report of a reliable evaluation of redox potentials of holo-sGDH and the observation of PQQ_{sem} in holo-sGDH.

Figure 1 Absorption spectra of holo-sGDH undergoing mediated CFCES

Spectra were measured with an electrode potential (E) varying between 197 and -153 mV. Corrections were made by subtracting the background spectra of $50 \mu\text{M}$ PES. Inset: the A_{338} and A_{485} of holo-sGDH as a function of E . The solid line represents the regression curve obtained by non-linear analysis, on the basis of eqn (5), for the 1E1E mechanism with $E_1^{\circ'} = 33$ mV and $E_2^{\circ'} = -12$ mV. The broken line represents the regression curve obtained by non-linear analysis, on the basis of eqn (8), for the 2E mechanism. Some irregularities occur in the wavelength region from 370–450 nm due to the correction for the strong absorption of PES. Abbreviations: mabs, milli-absorbance units; SHE, standard hydrogen electrode.

Figure 2 Auto-oxidation of glucose-reduced holo-sGDH

Spectrum A: reduced holo-sGDH generated by the addition of a sub-stoichiometric amount of glucose under anaerobic conditions. Spectrum B: after exposure of reduced holo-sGDH to air for 25 min. Spectrum C: after exposure of reduced holo-sGDH to air for 60 min. Inset: ESR signal observed under the conditions corresponding to those used for obtaining spectrum B.

Similar spectroelectrochemical experiments were performed for holo-X (Figure 3). In this case, as reported previously [18], the absorption band assigned to PQQ_{red} was situated at a much shorter wavelength than in holo-sGDH (Table 1), and overlapped with that assigned to the aromatic amino acids in the protein. In

Figure 3 Absorption spectra of holo-X undergoing mediated CFCES

Spectra were measured with an electrode potential (E) between 197 and -153 mV. Corrections were made by subtracting the background spectra of $50 \mu\text{M}$ PES. Inset: A_{307} of holo-X as a function of E . The line represents the regression curve obtained by non-linear analysis, on the basis of eqn (8), for the 2E mechanism with $E^{\circ'} = 37$ mV. Abbreviations: mabs, milli-absorbance units; SHE, standard hydrogen electrode.

contrast with holo-sGDH, no intermediate spectral species was observed, the spectra having an isosbestic point at 342 nm (Figure 3). This means that Ca^{2+} in the active site of holo-sGDH can stabilize PQQ_{sem} , at least from the thermodynamic viewpoint, which is consistent in that the absence of semiquinone has also been observed for a Ca^{2+} -lacking form of methanol dehydrogenase [22]. Therefore the spectral data were analysed based on the 2E mechanism. Non-linear regression analysis gave $E^{\circ'}$ as 37 mV, which is 26.5 mV more positive than that of holo-sGDH. Since the 2E mechanism was successfully applied to this system (Figure 3, inset), $E_2^{\circ'} - E_1^{\circ'}$ would be equal or larger than 100 mV (see the Experimental section), i.e. $E_1^{\circ'} \leq -10$ mV and $E_2^{\circ'} \geq 90$ mV ($K_{\text{sem}} \leq 0.02$). Judging from the potential shifts in $E_1^{\circ'}$ and $E_2^{\circ'}$ related to K_{sem} , the incorporation of Ca^{2+} in the active site of sGDH stabilizes PQQ_{sem} , apparently by interacting more strongly with PQQ_{sem} than with PQQ_{ox} and PQQ_{red} in the enzyme. Similarly, the fact that the two-electron redox potential ($E^{\circ'}$) of holo-sGDH was slightly lower than that of holo-X indicates that Ca^{2+} interacts more strongly with PQQ_{ox} than with PQQ_{red} in holo-sGDH.

Redox characteristics of free PQQ

Direct electrolysis of free PQQ in aqueous solution at conventional electrodes, such as glassy carbon, gold, platinum and carbon-fibre packed column electrodes exhibits quasi-reversible electron transfer, the exception being at mercury electrodes [23,24]. However, reversible electron transfer was obtained for free PQQ with CFCES using PES and/or PMS ($E_{\text{PMS}}^{\circ'} = 80$ mV) as mediators. The spectral data obtained at pH 7.0 are presented in Figure 4, with isosbestic points at 282 and 342 nm but with no absorption band that could be attributed to PQQ_{sem} . These results indicate that the electron transfer of free PQQ proceeds in the 2E mechanism without detectable formation of PQQ_{sem} , at least under these conditions (pH 7.0). Therefore, the spectral

Table 1 Redox properties and related parameters of PQQ under various conditions

The values of $E^{\circ'}$ are the means $\pm$ S.D. of the non-linear regression.

Species	Mechanism	$E^{\circ'}$ (mV)	$E_1^{\circ'}$ (mV)	$E_2^{\circ'}$ (mV)	K_{sem}	$A_{\text{red,max}}$	Buffer
Holo-sGDH	1E1E	10.5 ± 4	33 ± 4	-12 ± 4	5.8	338	pH 7.0
Holo-X	2E	37 ± 2	(≤ -10)	(≥ 90)	$(\leq 0.02)^{\dagger}$	307	pH 7.0
PQQ	2E	65 ± 2	$(15)^{\dagger}$	$(115)^{\dagger}$	(0.02) [24]	303	pH 7.0
PQQ- Ca^{2+}	1E1E	81 ± 6	113 ± 6	49 ± 6	12.1	319	pH 7.0, 1 M CaCl_2
PQQ- Mg^{2+}	2E	99 ± 2	—	—	≤ 1	334	pH 7.0, 1 M MgCl_2
PQQ- Na^+	2E	73 ± 2	—	—	≤ 1	303	pH 7.0, 3 M NaCl
PQQ- K^+	2E	84 ± 2	—	—	≤ 1	303	pH 7.0, 3 M KCl
PQQ	1E1E	-114 ± 6	-116 ± 6	-112 ± 6	0.9	319	pH 11.0
PQQ- Ca^{2+}	1E1E	-97 ± 5	-91 ± 5	-103 ± 5	1.6	339	pH 11.0, 1 M CaCl_2

$\dagger$ See the text.

Figure 4 Absorption spectra of free PQQ undergoing mediated CFCES

Spectra were measured with an electrode potential (E) between 247 and -103 mV. Corrections were made by subtracting the background spectra of $30 \mu\text{M}$ PMS and $20 \mu\text{M}$ PES. Inset: A_{303} of free PQQ as a function of E . The line represents the regression curve obtained by non-linear analysis on the basis of eqn (8) for the 2E mechanism with $E^{\circ'} = 65$ mV. Abbreviations: mabs, milli-absorbance units; SHE, standard hydrogen electrode.

data were analysed based on eqn (8). The value obtained for $E^{\circ'}$ (Table 1) is in good agreement with the value reported previously [24] ($E^{\circ'} = 65$ mV; digital simulation analysis of reversible cyclic voltammograms at a mercury electrode gave $E_1^{\circ'}$ and $E_2^{\circ'}$ as 15 and 115 mV respectively, and $K_{\text{sem}} = 0.02$ [24]), although this value is slightly more negative than that of another report ($E^{\circ'} = 90$ mV [20]).

To investigate the effect of Ca^{2+} on the redox reactions of free PQQ, cyclic voltammograms of PQQ were measured in the presence of 1.0 M CaCl_2 with a mercury electrode. However, the voltammograms showed complicated behaviour, most probably due to the adsorption of Ca^{2+} -chelated complex(es), as has been observed for other quinones [25]. Therefore the application of mediated CFCES to this system was attempted. Spectra of PQQ in the presence of 1.0 M Ca^{2+} as a function of E are shown in Figure 5. They show that Ca^{2+} induced a red shift in the absorption band of PQQ_{red} (to 319 nm), and the emergence of a broad band around 615 nm at about $E = 80$ mV. The

Figure 5 Absorption spectra of PQQ- Ca^{2+} undergoing mediated CFCES

Spectra were measured with an electrode potential (E) between 227 and -103 mV. Corrections were made by subtracting the background spectra of $30 \mu\text{M}$ PMS and $20 \mu\text{M}$ PES. Inset: A_{321} (●) and A_{612} (■) of PQQ- Ca^{2+} (1 M) as a function of E . The solid lines represent the regression curves obtained by non-linear analysis, on the basis of eqn (5), for the 1E1E mechanism with $E_1^{\circ'} = 113$ mV and $E_2^{\circ'} = 49$ mV. The broken line represents the regression curve obtained by non-linear analysis, on the basis of eqn (8), for the 2E mechanism. Abbreviations: mabs, milli-absorbance units; SHE, standard hydrogen electrode.

potential dependence of A_{321} and A_{612} , depicted in the inset of Figure 5, is indicative of PQQ_{sem} formation, probably in equilibrium with a diamagnetic quinhydron, which sandwiches Ca^{2+} present in the solution [20].

The (apparent) redox potentials were calculated to be $E_1^{\circ'} = 113$ mV and $E_2^{\circ'} = 49$ mV ($E^{\circ'} = 81$ mV, $K_{\text{sem}} = 12.1$) by analysis based on the 1E1E mechanism. When a solution of NaBH_4 -reduced PQQ in the presence of Ca^{2+} at pH 7.0 was exposed to air, the observed absorption spectra and ESR signal were compatible with those of PQQ_{sem} , although the ESR intensity of the solution was not strong enough to get the hyperfine structure, because of precipitation of the complex in aqueous solution at increased concentrations adjusted for ESR measurements. However, it is noteworthy that Ca^{2+} stabilizes PQQ_{sem} (and diamagnetic quinhydron) even at neutral pH.

Figure 6 shows that upon increasing the CaCl_2 concentration in the PQQ solution, $E_1^{\circ'}$ becomes more positive, and $E_2^{\circ'}$ shifts in

Figure 6 Ca²⁺ dependence of the one-electron redox potentials of free PQQ

The redox potentials E_2' (●) and E_2'' (■), at various concentrations of Ca²⁺, were evaluated similarly to those indicated in the legend to Figure 5, whereas those in the absence of Ca²⁺ were taken from the literature [24]. SHE, standard hydrogen electrode.

a negative direction. This means that Ca²⁺ interacts more strongly with PQQ_{sem} than with PQQ_{ox} and PQQ_{red}. Since a slightly positive shift was observed in the two-electron redox potential (E^0), Ca²⁺ interacts more strongly with PQQ_{red} than with PQQ_{ox}. Attempts to describe the Ca²⁺-concentration dependence of the redox potentials in a quantitative way, by using a model in which stoichiometric complexes of Ca²⁺ with PQQ_{ox}, PQQ_{sem} and PQQ_{red} were formed, were unsuccessful. This could be due to complications arising from multiple binding of Ca²⁺ ions to the redox species, perturbation of acid/base equilibria by Ca²⁺, and/or a drastic change in solvent property caused by the addition of CaCl₂. Therefore, since the effect of Ca²⁺ was much less at pH 11.0 than at pH 7.0 (Table 1), it is clear that the pH affects the interaction between Ca²⁺ and the redox species of PQQ.

When MgCl₂ was used instead of CaCl₂, no detectable stabilization of PQQ_{sem} occurred for PQQ in its free state, although a positive shift of E' and a red shift of the absorption band of PQQ_{red} were observed (Table 1). K⁺ and Na⁺ also induced the positive shift but not the red shift (Table 1).

DISCUSSION

The use of the CFCES method enabled, for the first time, determination of the effect of Ca²⁺ on the redox properties of PQQ, both in its free state and when bound to an enzyme; the latter by comparing the redox properties of holo-sGDH and holo-X. From the results obtained, it can be safely concluded that Ca²⁺ has a stabilizing effect on PQQ_{sem} in the enzyme as well as in its free state, since no detectable structural differences exist between holo-sGDH and holo-X [17,28]. Based on the effects on the redox potential of the steps concerned, this must be due to the stronger effect of Ca²⁺ on PQQ_{sem} than on PQQ_{ox} and PQQ_{red}. From structural data of methanol dehydrogenase [26,27] and sGDH [17,28], it is known that Ca²⁺ interacts with the carboxy group of PQQ at the 7-position, with the N-6 of its quinoline moiety and with the carbonyl group at the 5-position. Similar structures can be considered for the binding of Ca²⁺ with PQQ_{sem}, as shown in Figure 7. The stabilization of PQQ_{sem} is probably

Figure 7 Proposed structure of the PQQ_{sem}-Ca²⁺ complex in s-GDH

due to the compensating effect of the positive charge of Ca²⁺ on the negative charge of the C-5 carbonyl group. Similarly, Ca²⁺ could compensate the negative charge of the deprotonated 5-hydroxy group in PQQ_{red}, and could have a polarizing effect on the 5-carbonyl group in PQQ_{ox}. Thermodynamically, the latter seems to be more important than the former, since in sGDH, Ca²⁺ induces a negative shift in the E' value. The opposite effect was observed for free PQQ, i.e. a slight positive shift in E' caused by the presence of Ca²⁺. However, since univalent cations behave similarly (Table 1), the increase must be due to a general salt effect. This could indicate that the mode of binding of Ca²⁺ to PQQ in sGDH and in its free state is somewhat different. In this context, it should be remembered that, besides the ligands already mentioned, the two main-chain carbonyl O atoms of Gly-247 and Pro-248, and two water molecules are also ligands to Ca²⁺ in sGDH [28]. The additional ligands may also explain why holo-X has a very high affinity for Ca²⁺, whereas the effects on free PQQ are apparent only at very high Ca²⁺ concentration. The fact that Mg²⁺ did not stabilize PQQ_{sem} but gave a strong red shift for PQQ_{red} indicates that either the mode of binding of Ca²⁺ and Mg²⁺ to the redox species of free PQQ is different or that this is caused by the different properties of these bivalent cations. The negligible effect of Mg²⁺ on the spectral properties of PQQ_{ox} and PQQ_{red} in mGDH [29] suggests that binding of Mg²⁺ to PQQ in this enzyme and in its free state are also different.

Realizing the differences noted above, this casts some doubt on the reliability of the conclusions, with respect to the role of these bivalent cations, as deduced from biomimetic researches. In model studies with the trimethyl ester of N-1-methylated PQQ in aprotic solvents, the importance of the positive shift in E_1' by the addition of Ca²⁺ has been emphasized [30]. However, such an effect has been observed for quinones in general upon addition of cations, and an even larger positive shift was observed for E_2' , at least in aprotic solvents, which was due to the interaction of the fully reduced quinone dianions with cations [31]. Since Ca²⁺ gave a negative shift in the E_2' value for PQQ in the enzyme as well as in the free state, this seems a more noteworthy property to be emphasized. As mentioned above, the interaction of PQQ_{red} with Ca²⁺ in sGDH would be weakened by the competitive protonation of the negatively charged oxygen atom derived from the quinone carbonyl group (pK_a of the 5-hydroxy group in free PQQ_{red} being 8.5 [24]).

As illustrated by the dramatic differences in catalytic-centre activity of holo-sGDH and holo-X [12], Ca²⁺ has a large stimulating effect on the reductive half-reaction of sGDH with glucose. Since oxidation of the substrate occurs via hydride transfer [12], the stabilizing effect of Ca²⁺ on PQQ_{sem} does not explain the stimulation. This view is confirmed by the fact that Mg²⁺ did not stabilize PQQ_{sem} but is, nevertheless, the stimulating

bivalent cation in mGDH. At first sight, the slightly negative shift in the E° of sGDH, caused by Ca^{2+} , seems almost counterproductive. However, since the redox potential of glucose/gluconolactone is so low (-364 mV [32]), this decrease has an insignificant effect on the overall thermodynamics of the reaction. In conclusion, the stimulation of Ca^{2+} in the reductive half-reaction seems to be confined to improving certain kinetic steps in the reaction [12]; for example, the polarizing effect on the C-5 carbonyl group of PQQ_{ox} , thus facilitating hydride transfer from the substrate to the C-5 carbon atom (and facilitating adduct formation between the PQQ analogue and substrate in model studies [33]), and binding to the deprotonated 5-hydroxy group in PQQ_{red} , so facilitating tautomerization of C-5-reduced PQQ to PQQ_{red} in the enzyme (and the intramolecular rearrangement of the adduct complex taking place in model reactions).

In contrast with the conclusion made for the reductive half-reaction, since the oxidation of reduced cofactor-containing dehydrogenase normally occurs via the respiratory chain in one-electron steps, the stabilization of PQQ_{sem} by Ca^{2+} could be important for the oxidative half-reaction. From a thermodynamic point of view, the lowering of the E° of sGDH by Ca^{2+} would be convenient for the oxidative half-reaction, since the redox potential of cytochrome b_{562} is 170 mV, which shifts to 145 mV in the presence of sGDH [16]. However, the significance of Ca^{2+} seems to relate to kinetic aspects also, e.g. by protecting PQQ_{sem} in sGDH against attack by O_2 . In this context, it should be noted that, although slow auto-oxidation was observed here for reduced sGDH, no significant turnover of glucose occurred under aerobic conditions in contrast to mGDH [29]. This could be because Mg^{2+} does not stabilize PQQ_{sem} , whereas Ca^{2+} does. Since sGDH has now replaced glucose oxidase in several applications as a sensor for diagnostic purposes, mainly because it is insensitive to the interfering effect of O_2 on glucose determinations [15], this provides an additional stimulus for studies on the stabilizing effect of Ca^{2+} on PQQ_{sem} .

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