Ca^{2+}-Assisted, Direct Hydride Transfer, and Rate-Determining Tautomerization of C5-Reduced PQQ to $PQQH_2$, in the Oxidation of β-D-Glucose by Soluble, Quinoprotein Glucose Dehydrogenase

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ABSTRACT: Spectral and kinetic studies were performed on enzyme forms of soluble glucose dehydrogenase of the bacterium *Acinetobacter calcoaceticus* (sGDH) in which the PQQ-activating Ca^{2+} was absent (Holo X) or was replaced with Ba^{2+} (Ba-E) or in which PQQ was replaced with an analogue or a derivative called "nitroPQQ" (E-NPQ). Although exhibiting diminished rates, just like sGDH, all enzyme forms were able to oxidize a broad spectrum of aldose sugars, and their reduced forms could be oxidized with the usual artificial electron acceptor. On inspection of the plots for the reductive half-reaction, it appeared that the enzyme forms exhibited a negative cooperativity effect similar to that of sGDH itself under turnover conditions, supporting the view that simultaneous binding of substrate to the two subunits of sGDH causes the effect. Stopped-flow spectroscopy of the reductive half-reaction of Ba-E with glucose showed a fluorescing transient previously observed in the reaction of sGDH with glucose-1-*d*, whereas no intermediate was detected at all in the reactions of E-NPQ and Holo X. Using hydrazine as a probe, the fluorescing C5 adduct of PQQ and hydrazine was formed in sGDH, Ba-E, and Holo X, but E-NPQ did not react with hydrazine. When this is combined with other properties of E-NPQ and the behavior of enzyme forms containing a PQQ analogue, we concluded that the catalytic potential of the cofactor in the enzyme is not determined by its adduct-forming ability but by whether it is or can be activated with Ca^{2+}, activation being reflected by the large red shift of the absorption maximum induced by this metal ion when binding to the reduced cofactor in the enzyme. This conclusion, together with the observed deuterium kinetic isotope effect of 7.8 on transient formation in Ba-E, and that already known on transient decay, indicate that the sequential steps in the mechanism of sGDH must be (1) reversible substrate binding, (2) direct transfer of a hydride ion (reversible or irreversible) from the C1 position of the β-anomer of glucose to the C5 of PQQ, (3) irreversible, rate-determining tautomerization of the fluorescing, C5-reduced PQQ to $PQQH_2$ and release (or earlier) of the product, D-glucono-δ-lactone, and (4) oxidation of $PQQH_2$ by an electron acceptor. The PQQ-activating Ca^{2+} greatly facilitates the reactions occurring in step 2. His144 may also play a role in this by acting as a general base catalyst, initiating hydride transfer by abstracting a proton from the anomeric OH group of glucose. The validity of the proposed mechanism is discussed for other PQQ-containing dehydrogenases.

Soluble quinoprotein (PQQ-containing) glucose dehydrogenase of the bacterium *Acinetobacter calcoaceticus* (sGDH,[1] EC 1.1.99.17) converts a broad spectrum of aldose sugars into the corresponding lactones which are hydrolyzed to the aldonic acids (D-glucose into D-glucono-δ-lactone, which is hydrolyzed to gluconic acid). In view of its high turnover number (*1*) and its broad artificial electron acceptor specificity (*2*−*4*), the enzyme is well suited for analytical applications, exemplified by its use as a sensor in glucose test strips and biosensors fabricated for diabetic patients (*5*). The enzyme consists of two identical subunits (*6*), six Ca^{2+} ions (*7*, *8*), and two PQQ molecules (*1*). Four of the Ca^{2+} ions are involved in dimerization of the subunits, two in activating the two PQQ molecules (*9*).

Catalysis by the enzyme under turnover conditions proceeds according to a ping-pong mechanism in which substrate inhibition by glucose and a negative cooperativity effect are displayed (*10*). The latter can be described by two sets of kinetic parameters, one set applying at low concentrations of glucose and the other at high concentrations. In view of the high k_{cat} values that were found [1600 and 18 000 s^{-1}, respectively (*7*)], catalysis by sGDH is extremely efficient, especially when the cooperativity effect is operational. The underlying mechanism for this behavior is at present unknown. Stopped-flow spectroscopy of the oxidation of glucose-1-*d* by sGDH revealed that under noncooperativity conditions, a reversible reaction occurs between the enzyme and substrate, leading to the formation of a fluorescing intermediate [with a deuterium kinetic isotope effect (KIE) of 1.5] which decomposes irreversibly to the reduced enzyme and gluconolactone (*7*). The decomposition step, having a

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[1] Abbreviations: sGDH, soluble quinoprotein glucose dehydrogenase; Holo X, sGDH in which the activating Ca^{2+} is lacking; Ba-E, hybrid form of sGDH in which dimerization and activation are carried out by Ca^{2+} and Ba^{2+}, respectively; NPQQ, nitroPQQ; E-NPQ, sGDH in which PQQ is replaced with NPQQ; KIE, deuterium kinetic isotope effect.

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KIE of 6.1, appeared to be the rate-determining step, not only for the reductive half-reaction but also under turnover conditions in the assay with *N*-methylphenazonium methyl sulfate (PMS). The simplest kinetic mechanism (Scheme 1)

Scheme 1: Previously Proposed Scheme for the Kinetic Mechanism of sGDH

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E'P$$

in agreement with this assumes that a reversible reaction between enzyme (E) and substrate (S) leads to formation of the fluorescing transient (ES) which decomposes irreversibly to reduced enzyme (E′P). Two different chemical mechanisms were proposed (*7*) which can explain this behavior of sGDH. The first is covalent catalysis, consisting of the following steps: enzyme reacting with glucose giving a fluorescing complex (the adduct of glucose and PQQ, the OH group at the C1 position of glucose reacting with the C5 carbonyl group of PQQ); and an irreversible, intramolecular rearrangement (an addition−oxidation−elimination mechanism) converting the complex into reduced enzyme, containing $PQQH_2$, and gluconolactone, the rearrangement being the rate-determining step. The second is direct hydride transfer, proceeding as follows: a reversible process in which glucose binds to the enzyme after which a hydride ion coming from the C1 position of glucose reduces PQQ at the C5 position, forming a fluorescing species; and tautomerization of this species in an irreversible, rate-determining step to $PQQH_2$. The difficulty identifying the nature of the fluorescing transient,[2] the uncertainty of whether a C−H bond breaking step occurs in its formation (the KIE of 1.5 is close to the upper limit of a secondary KIE), and the absence of evidence for the step in which the substrate is actually oxidized prevented a conclusion from being drawn on the mechanism at that time.

Due to the high turnover number of sGDH, it was technically impossible to determine the individual numerical rate constants for undeuterated substrates with our stopped-flow equipment (*7*). To enable such studies, therefore, in the first instance, we focused on how to circumvent this problem. In this context, we attempted to prepare artificial enzyme forms with a substantially lower catalytic performance than their natural counterpart. For this purpose, a previously described enzyme form, the so-called Holo X (*9*), in which the PQQ-activating Ca^{2+} ions are lacking, was investigated. In addition, a new hybrid enzyme form (denoted Ba-E) was constructed in which the dimerization of the subunits is furnished by Ca^{2+} but the activation of PQQ by Ba^{2+}. PQQ analogues (*11*) were tested for their ability to bind to the apoenzyme and holoenzymes which were formed for their performance in catalyzing glucose oxidation. Derivatization of PQQ by nitration has been described (*12*). Although the structure of the derivative has not been elucidated yet, an enzyme form (denoted E-NPQ) in which PQQ is replaced by "nitroPQQ" (NPQQ) was interesting to investigate since it was found that the derivative is not susceptible to nucleophilic attack, i.e., does not form adducts at the C5 position. Since the adduct-forming ability of the cofactor could be an important parameter for discriminating between the two possible mechanisms, nucleophilic, non-substrate compounds were tested, eventually (methyl)-hydrazine appearing to be a suitable probe for this.

The preliminary characterization of the artificial enzyme forms described here provided insight into those properties of PQQ and the activating metal ion that is decisive for the mechanism of action of sGDH. Furthermore, it showed that Ba-E was suited to being used in stopped-flow experiments with undeuterated substrates. Since the exploratory investigations on the pre-steady-state kinetics of Ba-E provided one of the clues in deciding which of the two proposed mechanisms is correct, this work is also presented here.

EXPERIMENTAL PROCEDURES

NitroPQQ (NPQQ) Synthesis. PQQ (15 mg) was dissolved in 5 mL of nitration agent (a mixture of equal volumes of concentrated H_2SO_4 and HNO_3), and the mixture was incubated on ice for 15 min. Sep-Pak Plus tC_{18} cartridges (Waters) were washed with 10 hold-up volumes of methanol (HPLC-grade) and subsequently with the same volume of water (milliQ-quality). The incubation mixture was diluted 10 times with water and brought to the pretreated C_{18} cartridges. The cartridges were washed with water, and the orange-colored NPQQ was eluted with methanol. The NPQQ-containing solution was brought to a reversed-phase column (2 cm × 30 cm, containing preparative C_{18}, 125 Å pore size, particle size of 55−105 μm, from Waters) pretreated with methanol/water washings as described for the cartridges. The column was washed with water (200 mL at a flow rate of 3.0 mL/min) and a linear gradient of 12.5 mM potassium phosphate buffer (pH 2.0) and 12.5 mM potassium phosphate buffer (pH 8.0) (200 mL at a flow rate of 3.0 mL/min) applied. The absorbance of the eluate was monitored with a photodiode array detector. NPQQ eluted at about pH 7. On the basis of the perfect overlay of the spectra taken by the detector throughout the peak corresponding to the eluting NPQQ, the compound was pure.

Enzyme Preparations. Apo-sGDH was prepared from the *Escherichia coli* recombinant strain as described previously (*1*). Holo X was prepared (*9*) from apo-sGDH by converting the latter into "dimer preparation" (the procedure consisting of removal of any adhering Ca^{2+}, other than that involved in dimerization, which could lead to activation of the subsequently added cofactor) and adding PQQ dissolved in Ca^{2+}-free buffer in an amount equimolar to the subunit concentration. Ca^{2+}-free buffer (*9*) was prepared in the following way; 400 mL of 20 mM MOPS buffer (pH 7.0) was gently shaken for 2 days with 10 g of Serdolit ion exchanger (Na^+ form, SERVA), after which filtration gave the indicated buffer. Ba-E preparations were prepared by adding $BaCl_2$ (1 mM final concentration) and PQQ (equimolar to the subunit concentration) to the dimer preparation, and incubating the mixture for 30 min. E-NPQ and the holoenzyme forms of the PQQ analogues were prepared in the same way except that $BaCl_2$ and PQQ were replaced with $CaCl_2$ and NPQQ or the analogue, respectively.

[2] Since C5-reduced PQQ is in fact an adduct of PQQ and a hydride ion, it can be anticipated that knowledge of the spectral properties of the intermediate will not enable discrimination between the two possibilities.

Standard Assay. The assay is based on determining the reduction rate of 2,6-dichlorophenolindophenol (DCPIP), using PMS as the primary electron acceptor. The assay mixture was prepared by mixing the following solutions (the sequence of addition as indicated here): 840 μL of 50 mM potassium phosphate buffer (pH 7.0), 50 μL of 1 M glucose, 50 μL of 1 mM DCPIP, 50 μL of 20 mM PMS, and 10 μL of an enzyme solution containing the appropriate amount of enzyme activity units (*1*).

Pre-Steady-State Measurements. The experiments were performed at 20 °C with a stopped-flow reaction analyzer (SX-18MV) from Applied Photophysics. To detect any transients in the reductive half-reaction, Ba-E, Holo X, or E-NPQ (30 μM) was mixed with glucose (50 mM) and the mixture monitored for changes in fluorescence intensity (excitation at 360 nm, emission at 470 nm) and absorbance intensity (absorption spectra taken from 270 to 600 nm with the photodiode array spectrophotometer at time intervals of 10 ms). To determine the mechanism of Ba-E, the traces representing the formation and decay of the fluorescing intermediate observed in its reaction with substrates were fitted with the KINSIM/FITSIM programs (*13*−*15*) as well as with the method previously used (*7*), applying equations derived from the mechanisms presented in Schemes 1 and 2. To generate the experimental data, enzyme concentrations

Scheme 2: Newly Proposed Scheme for the Kinetic Mechanism of sGDH

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E'P \xrightarrow{k_5} E'' + P$$

were used in the range of 10−40 μM and substrate concentrations in the range where the cooperativity effect is still absent.

Sugar solutions were allowed to mutarotate to the equilibrium mixture of the α- and β-anomers before using them in kinetic experiments. All kinetic data are expressed in terms of analytical sugar concentrations, although it is known that sGDH has an absolute preference for the anomer which has the same H/OH configuration as β-D-glucose at the C1 position (*7*).

Spectrophotometric Measurements. Fluorescence and absorption spectra were recorded with a Shimadzu RF-5001PC and a Hewlett-Packard 8453 diode array spectrophotometer, respectively. To investigate the adduct-forming ability of the cofactor in the enzyme forms, aliquots of a freshly prepared solution of hydrazine in Ca^{2+}-free buffer were added.

RESULTS

SGDH in Which the Activating Ca^{2+} Is Lacking (Holo X). As has been reported previously (*1*), upon addition of glucose to Holo X its absorption spectrum slowly changes. Although the original and final spectra resemble those of free PQQ and $PQQH_2$, respectively, the resemblance is not caused by dissociation of the cofactor since the fluorescence of PQQ in Holo X is the same as in sGDH (about 10% of that of free PQQ), and gel filtration of Holo X does not remove the cofactor. When the reaction of glucose with Holo X was followed in the stopped-flow apparatus, no spectral intermediates were detected, neither by rapid scanning nor by monitoring for fluorescence. Just as for sGDH under steady-state conditions (*10*), Holo X exhibited a negative cooperativity effect in the reductive half-reaction with glucose (Figure 1). Although the overall rate of the reductive half-reaction of Holo X was only 0.05% of that of sGDH under similar conditions (Table 1), surprisingly the switch from the mechanism operating at low to that operating at high concentrations occurred at the same order of magnitude as has been found for sGDH under turnover conditions (*10*), not only for Holo X but also for Ba-E and E-NPQ (Figure 1). All these enzyme forms oxidized the same spectrum of sugars, although the ratios of the specificity constant values were not constant (data not shown).

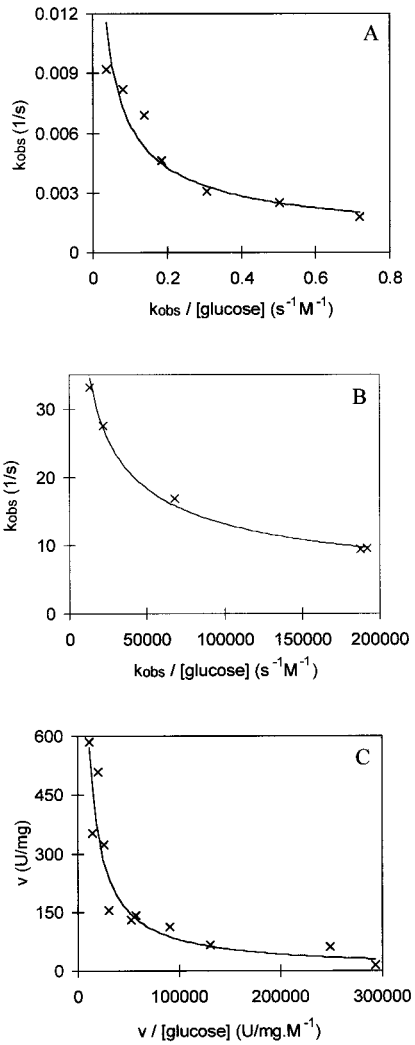


FIGURE 1: Eadie−Hofstee plots for the reductive half-reaction of Holo X (A) and Ba-E (B) and for the steady-state kinetics of E-NPQ (C) with glucose. All reactions were carried out in Ca^{2+}-free buffer, except in the case of Ba-E where it contained 1 mM $BaCl_2$. Enzyme concentrations varied between 5 and 20 μM and the glucose concentrations between 10 μM and 0.5 M. The progression of the reaction for Holo X and Ba-E was monitored with the rapid-scan and with the fluorescence spectrophotometer device, respectively. Panel B shows the plot for $k_{obs,1}$, but that for $k_{obs,2}$ (see the legend of Figure 3 for the nomenclature) exhibited a similar profile. The steady-state kinetics of E-NPQ were determined in the standard assay with a varying glucose concentration.

SGDH in Which the Activating Ca^{2+} Is Replaced by Ba^{2+} (Ba-E). As has been demonstrated by gel filtration (*9*), no stable dimer is formed when Ba^{2+} is added to a monomeric

Table 1: Activities of Different Enzyme Forms with Glucose in the Reductive Half-Reaction and in the Standard Assay

enzyme	k_{obs}[a] (s^{-1})	standard assay[b] (units/mg)
sGDH	nd[c]	6400
Holo X	0.3	ld[d]
Ba-E	64	100
E-NPQ	340	320

[a] The k_{obs} values were determined with the stopped-flow technique by mixing the enzyme form with glucose (1 mM). The reaction of Holo X was monitored at 307 nm with 2.5 s time intervals, that of Ba-E by following the fluorescence intensity, and that of E-NPQ using the photodiode array spectrophotometer. Ca^{2+}-free buffer was used, except in the case of Ba-E where it was supplemented with 1 mM $BaCl_2$. [b] Rates in the standard assay were measured using the appropriate enzyme concentration. [c] nd, cannot be determined due to the high turnover number. [d] ld, low but detectable activity.

enzyme preparation. In agreement with this, when a monomer preparation (prepared from apo-sGDH; see ref *9*) was incubated with PQQ and Ba^{2+}, no activity was found in the standard assay and the fluorescence of PQQ hardly decreased, indicating that it was not bound. However, upon titration of Holo X (prepared with Ca^{2+}) with a $BaCl_2$ solution in the presence of glucose, the following observations were made. At sub-millimolar $BaCl_2$ concentrations, spectra indicated that only Holo X_{red} was present, that at a concentration of 1 mM 50% Holo X_{red} and 50% of an enzyme form spectrally resembling $sGDH_{red}$ were present, and that at 20 mM the previous ratio had changed to 10:90. This indicated that the affinity of Holo X for Ba^{2+} is rather low, as was confirmed by gel filtration of Ba-E, showing that it reverted to Holo X under this condition. However, the typical spectrum of $sGDH_{red}$ obtained at a sufficiently high $BaCl_2$ concentration indicates that Ba^{2+} is able to induce the same spectral red shift as Ca^{2+} (*1*) with respect to the maximum of the absorption spectrum of $PQQH_2$ in the enzyme {although it should be noted that the maximum of the absorption spectrum of Ba-E_{red} is 2 nm blue-shifted as compared to the value [338 nm (*9*)] for $sGDH_{red}$}.

On testing several nucleophilic compounds, we found that hydrazine gave competitive inhibition with respect to glucose in the reaction with sGDH. Since not only glucose but also gel filtration completely abolished the inhibition displayed by the hydrazine-treated enzyme, this indicated that a dissociable complex was formed between the enzyme and hydrazine. Fluorescence spectroscopy revealed that a slow increase in fluorescence intensity occurred when hydrazine was added to Holo X, Ba-E, or sGDH, the intensity eventually becoming 50 times higher than the original value of sGDH. Since the spectral characteristics of hydrazine-treated sGDH (maxima $\lambda_{exc} = 370$ nm, $\lambda_{em} = 450$ nm; see Figure 2) were similar to those of hydrated, free PQQ (maxima $\lambda_{exc} = 371$ nm, $\lambda_{em} = 464$ nm) (*16*) and a PQQ adduct in methanol dehydrogenase (MDH) (maxima $\lambda_{exc} = 375$ nm, $\lambda_{em} = 446$ nm) (*17*), this strongly suggested that an adduct was formed between hydrazine and PQQ at the C5 position. Structural investigations on crystals of sGDH soaked in a methylhydrazine-containing solution have, meanwhile, proven that the hydrazine is indeed bound to PQQ in the enzyme in this way (*18*).

Ba-E exhibited only 1.5% of the activity of sGDH in the standard assay (Table 1). Since this low rate stems from the low overall rate of the reductive part of the catalytic cycle

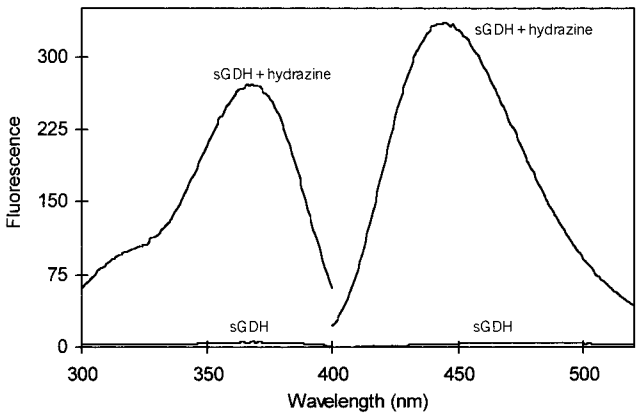


FIGURE 2: Fluorescence spectra of sGDH and sGDH treated with hydrazine. The fluorescence excitation and emission spectra (excitation at 360 nm and emission at 470 nm) were recorded for 10 μM sGDH in 20 mM MOPS/NaOH buffer (pH 7.0) and after addition of hydrazine (to a final concentration of 20 μM) and incubation for 45 min.

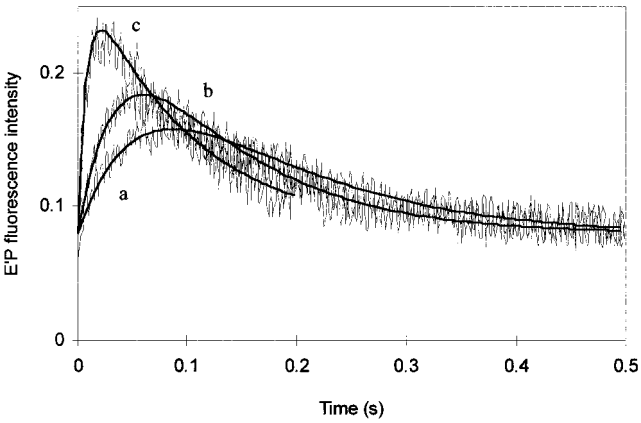


FIGURE 3: Formation and decay of the transient (E′P) in the reductive half-reaction of Ba-E with glucose, as measured with stopped-flow fluorescence spectroscopy. Experiments were carried out by mixing the contents of syringe 1 (containing glucose in Ca^{2+}-free buffer) with those of syringe 2 (containing Ba-E in the same buffer with 1 mM $BaCl_2$). Ba-E (5.0 μM) was mixed with the following concentrations of glucose: 50 (a), 100 (b), and 500 μM (c). The traces represent the fluorescence intensity observed after mixing (excitation at 360 and emission at 470 nm). The traces were fitted (drawn line) with the double exponential equation $y = A_1 \times \exp(-k_{obs,1}t) + A_2 \times \exp(-k_{obs,2}t) + E$, where y is the fluorescence intensity, A is the amplitude of the signal, k_{obs} is the observed rate, t is the time axis, and E is the end point.

(Table 1), this enzyme form seemed to be suited for those stopped-flow experiments which can technically not be performed with sGDH. Just as for sGDH with glucose-1-*d* (*7*), a fluorescing transient was observed in the reaction of Ba-E with glucose, the height and the shape of the traces being affected by the concentrations as depicted in Figure 3. Using the k_{obs} values derived from the traces generated by glucose and glucose-1-*d*, fitting according to the mechanism shown in Scheme 1 revealed a very large KIE value for the formation rate of the fluorescing transient [when this is expressed as a K_M value, as has been done in ref *7*, a KIE value of 39.5 was calculated for Ba-E whereas a value of 1.5 has been found for normal sGDH (*7*)]. Together with other arguments, this leads to the conclusion that the mechanism proceeds according to the direct hydride transfer mechanism (see below). As a consequence, the previously proposed mechanism (Scheme 1) is too simple and the most

Table 2: Numerical Rate Constants for Ba-E with Various Substrates and Conditions[a]

substrate (buffer)	$k_1k_3/(k_2 + k_3)$ (mM^{-1} s^{-1})	k_5 (s^{-1})
glucose (MOPS at pH 7.0)	532 ± 6	10.0 ± 0.1
glucose-1-*d* (MOPS at pH 7.0)	68 ± 1	2.60 ± 0.01
KIE	7.8	3.8
glucose (MES at pH 4.5)	481 ± 14	5.71 ± 0.18
glucose (Tris-HCl at pH 9.0)	5679 ± 257	7.90 ± 0.12
mannose (MOPS at pH 7.0)	12.3 ± 0.1	15.6 ± 0.1

[a] The values were calculated by fitting with an equation for the newly proposed kinetic mechanism (Scheme 2).

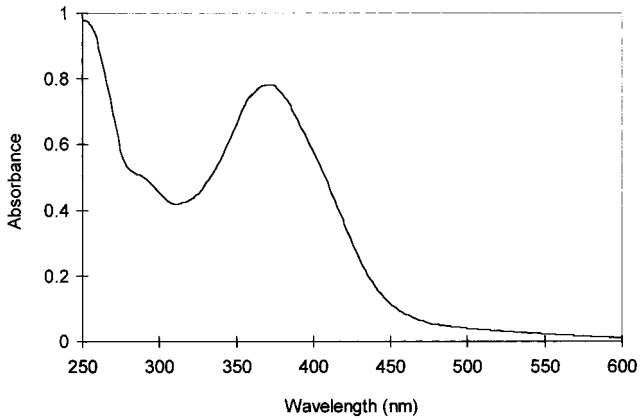


FIGURE 4: Absorption spectrum of NPQQ. The absorption spectrum of 30 μM NPQQ in Ca^{2+}-free buffer was recorded. The molar absorption coefficient of NPQQ at 370 nm is 24000 $M^{-1}cm^{-1}$ (derived from the titration of apo-sGDH with NPQQ, the amount of E-NPQ formed determined with the standard assay).

logical explanation of the observed phenomena is the mechanism depicted in Scheme 2 (see below), in which E′P stands now for the fluorescing transient and E″ for the reduced enzyme. On the basis of this mechanism, the rate of transient formation is given by $k_1k_3/(k_2 + k_3)$ and the rate of decay by k_5. Values for these kinetic parameters derived for different substrates and conditions are given in Table 2.

SGDH in Which PQQ Is Replaced with NitroPQQ (E-NPQ). The structure of nitroPQQ (NPQQ) is under investigation. The procedure described here for preparing the derivative was reliable, as judged from the fact that it was obtained in good yield on several occasions and by several persons. A preparation of NPQQ was stable on storage, and no free radical was observed in it with EPR spectroscopy. A solution of NPQQ does not fluoresce, and in accordance with this, varying the temperature did not affect the absorption spectrum [as it does for a solution of PQQ since the ratio of PQQ to the fluorescing, hydrated PQQ is shifted by this (*16*)]. The absorption spectrum is clearly distinct from that of PQQ and has a maximum at 370 nm (Figure 4). NPQQ is more loosely bound to the apoenzyme than PQQ since E-NPQ partially lost NPQQ on gel filtration and titration of the apoenzyme with NPQ enabled determination of the dissociation constant (5 μM), whereas this value is so low in the case of PQQ that it cannot be measured (*9*). Also for NPQQ, Ca^{2+} appears to be able to induce the typical red shift (Figure 5) when activating the cofactor in the enzyme, as judged from the maximum at 400 nm for E-NPQ_{ox} and that at 420 nm for E-NPQ_{red} (glucose addition to the "Holo X form" of E-NPQ_{ox} did not result in any spectral changes). The activity obtained in the standard assay (5% of that of

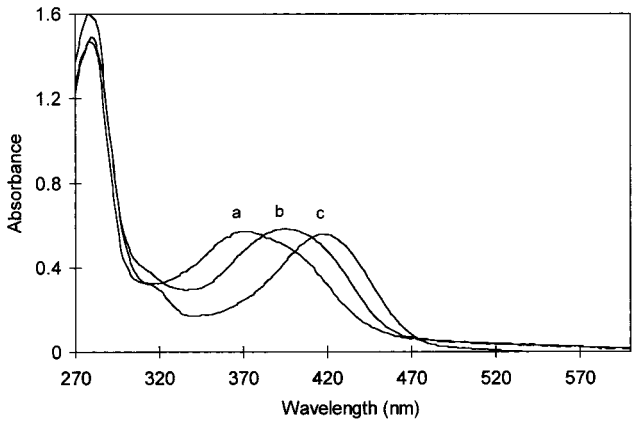


FIGURE 5: Absorption spectra of E-NPQ enzyme forms. Absorption spectra were taken in Ca^{2+}-free buffer of apo-sGDH (dimer preparation, 16 μM) after addition of a stoichiometric amount of NPQQ, yielding the Holo X form of E-NPQ (a); then after the solution was brought to 1 mM $CaCl_2$, yielding E-NPQ (b); and subsequently after the solution was brought to 10 mM glucose, yielding reduced E-NPQ (c).

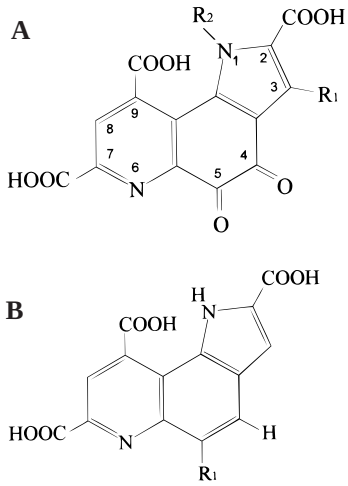


FIGURE 6: Structures of PQQ and its analogues (A) and of PQ and its analogues (B). (A) For PQQ, R1 and R2 = H. For 3-methylPQQ, R1 = CH_3 and R2 = H. For 3-propylPQQ, R1 = $CH_2CH_2CH_3$ and R2 = H. For N1-methylPQQ, R2 = CH_3 and R1 = H. For N1-ethylPQQ, R2 = CH_2CH_3 and R1 = H. (B) For PQ, R1 = H. For 5-hydroxyPQ, R1 = OH. For 5-methoxyPQ, R1 = OCH_3.

sGDH, Table 1) is not due to traces of contaminating, underivatized PQQ since the spectrum of glucose-reduced E-NPQ (Figure 5) is completely different from that of $sGDH_{red}$. E-NPQ does not fluoresce and does not react with hydrazine, and no intermediate was observed in its reaction with glucose, neither by absorption nor by fluorescence spectroscopy.

SGDH in Which PQQ Is Replaced with One of Its Analogues. All PQQ analogues (Figure 6A) and also 5-hydroxyPQ (Figure 6B) became bound to the apoenzyme [as has also been found in the case of quinohemoprotein alcohol dehydrogenase (*19*)], since the enzyme forms did not lose the analogues or 5-hydroxyPQ on gel filtration and the fluorescence of the analogues was significantly lower when they were bound to the enzyme than when in the free form. However, probably due to its abnormal structure, precluding an appropriate fit, activation by Ca^{2+} was not observed in the case of 3-propylPQQ, the absorption spectra of this enzyme form being similar to those of Holo X, and the activity in the standard assay being only 0.3% of that of

Table 3: Activities of Enzyme Forms Containing a PQQ Analogue[a]

PQQ analogue	activity (%)	PQQ analogue	activity (%)
3-methylPQQ	11	N1-methylPQQ	10
3-propylPQQ	0.3	N1-ethylPQQ	13

[a] Apo-sGDH was reconstituted with PQQ analogues in the presence of 1 mM $CaCl_2$ in 20 mM MOPS/NaOH (pH 7.0), and activities were measured in the standard assay. The activity of sGDH under this condition is taken as 100%.

sGDH (Table 3). On the other hand, the enzyme forms prepared from 3-methylPQQ and N1-methylPQQ behaved normally, as judged from the absorption spectra being similar to those of sGDH, and the relatively good activities in the standard assay [11 and 10% of the value of sGDH, respectively (Table 3)].

DISCUSSION

Comparison of the Performance of the Artificial Enzyme Forms with That of sGDH. In contrast to some other PQQ-containing dehydrogenases, e.g., the alcohol dehydrogenase from *Pseudomonas aeruginosa* (*20*), the activating Ca^{2+} ion in sGDH is not crucial for binding of PQQ, as deduced from the fact that PQQ remained bound on gel filtration of Holo X. Thus, this type of Ca^{2+} ion is primarily involved in activating PQQ in sGDH. The importance of this is illustrated by the very low reaction rate of Holo X in the reductive half-reaction and in the standard assay. Since PQQ in Holo X is easily converted into $PQQH_2$ by reducing substances, the very low rate (and the absence of a detectable intermediate in the reductive half-reaction, caused by a very low rate of fluorescing intermediate formation) must be due to the nearly complete inability of Holo X to become reduced by glucose. Thus, the absence of the PQQ-activating Ca^{2+} apparently results in the nearly complete inability of Holo X to oxidize substrate. These observations are in line with the results from model studies on the reaction of PQQ derivatives with alcohols in the presence of bivalent metal ions, showing that Ca^{2+} accelerates alcohol oxidation (*21*).

Since Ba-E was found to be active, it can be concluded that a metal ion which is inactive in dimerization of the subunits can nevertheless activate PQQ in sGDH. In view of the different ligands involved in binding of the "two different types of Ca^{2+}" in sGDH, this is not surprising. The decrease in activity of nearly 2 orders of magnitude is surprising, however, since substitution of the activating Ca^{2+} with Sr^{2+} did not change the specific activity of the enzyme (*10*). Substitution of Ca^{2+} with Ba^{2+} and Sr^{2+} has also been carried out for MDH (*22*). However, for this PQQ-containing enzyme, the substitutions led to strongly increased maximal rate and K_M values under steady-state conditions. Since the same fluorescing species was seen in the reaction of Ba-E and sGDH with glucose and no other intermediate was observed, both enzymes have the same overall mechanism. However, from the data in Table 2, it appears that the KIE values for the formation and decay rates of the fluorescing intermediate are 7.8 and 3.8, respectively, whereas these are 1.5 and 6.1 for sGDH (*7*). Thus, the difference between Ba^{2+} and Ca^{2+} in activating the enzyme concerns not only the rate-determining step (k_5) but also the two C−H bond breaking steps taking place in the reaction mechanism (see below). Together with the fact that the activating Ca^{2+} is bound to PQQ in an identical way in sGDH (*8*, *23*) and in MDH (*24*, *25*), this means that Ba^{2+} has the potential to activate PQQ, but apparently subtle differences in the active site of PQQ-containing enzymes determine the way and the extent to which the activation is expressed.

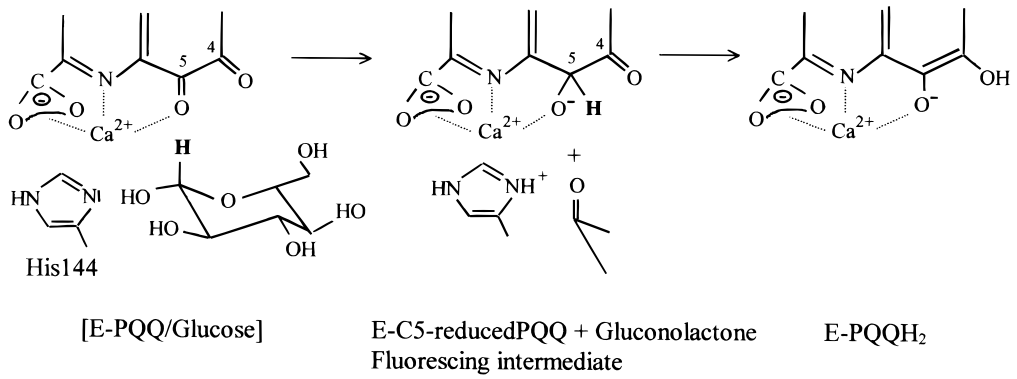
Comparison of the kinetic parameter values of glucose and mannose (Table 2) indicates that the k_5 values do not differ so much, whereas a large difference exists between the values for the rates of fluorescent intermediate formation. It has been previously concluded that the k_{cat} values for the substrates (the β-anomers of aldoses that exhibit a $^4C_1$-chair conformation, i.e., β-D-glucose and its structural homologues) of sGDH are the same, although great variation exists in the values for the specificity constant (*7*). Thus, this is another indication that Ba-E and sGDH have the same overall mechanism. It has been reported that sGDH has a pH optimum of about 7 for the reductive half-reaction as well as for assays in which an artificial electron acceptor is used which reoxidizes the reduced enzyme at a rate which is higher than that of the rate-determining step of the reductive half-reaction (*7*). The data in Table 2 show that Ba-E exhibits more or less similar behavior since for the three pH values that were tested, a slight optimum is observed for k_5 at pH 7 [the more pronounced optimum obtained for sGDH (*7*) is most probably due to the use of potassium phosphate buffer since it is known to enhance the value of the rate-determining step by a factor 3.5 in comparison with "Good" buffers such as Tris or MOPS (*10*)]. On the other hand, the values for the rate of fluorescent intermediate formation increases with pH, especially when going from pH 7 to 9, suggesting that an amino acid residue could be involved in a catalytic step in this process. In the absence of such data for sGDH, it cannot be decided yet whether this is unique for Ba-E.

The properties exhibited by E-NPQ show that the inability of a cofactor to form adducts does not result in insignificant catalytic activity or in the inability to become activated by Ca^{2+}. Thus, activation and activity seem to be correlated, as confirmed by several other examples: the enzyme form containing 3-methylPQQ shows good activity and spectra indicating activation; the enzyme form containing 3-propylPQQ shows neither of these, although the free cofactor is normally hydrated; Holo X shows very low activity because PQQ in it is not activated. In view of the substantial activity in the assay, and the absence of indications that the overall mechanism is basically different, the inability to detect an intermediate in the reaction of E-NPQ with glucose suggests that once the adduct is formed (from NPQ and the hydride ion, i.e., C5-reduced NPQQ; see below), it rapidly tautomerizes to $NPQQH_2$. The low stability of the adduct proposed here probably also applies in other cases, explaining why free NPQQ does not form an adduct with H_2O, and NPQQ in E-NPQ does not react with hydrazine.

All PQQ analogues and NPQQ were bound to the apoenzyme. Since also 5-hydroxyPQ was able to do so, apparently an intact *o*-quinol moiety is not required for binding. In addition, the spectral red shift accompanying the conversion of Holo X_{red} to $sGDH_{red}$ was also observed when Ca^{2+} was added to the complex of the apoenzyme and 5-hydroxyPQ, not with that containing 5-methoxyPQ.[3] Thus,

[3] A. J. J. Olsthoorn and J. A. Duine, unpublished results.

Scheme 3: Chemical Mechanism of Glucose Oxidation by sGDH



for the spectral red shift to occur, an unmodified OH group at C5 is required, in line with the fact that Ca^{2+} is bound to the C5 group of reduced PQQ, in MDH (*24*, *25*) as well as in sGDH (*23*). Most probably, the red shift originates from replacement of the proton in the OH group at C5 of $PQQH_2$ with Ca^{2+}, Ca^{2+} acting as a strong acid. Since the value of the rate-determining step in the conversion of glucose-1-*d* with Ba-E was much lower than that for sGDH, it is clear that the type of metal ion affects the decay rate of the intermediate. However, although Ca^{2+} is apparently better than Ba^{2+} in enhancing this, as illustrated by Holo X, reduction of PQQ to $PQQH_2$ can proceed in the absence of a bivalent metal ion. On the other hand, as discussed above, the metal ion has a tremendous effect on the formation rate of the intermediate in the reductive half-reaction, possibly by polarizing the carbonyl group of PQQ at C5. However, the position of the cofactor in the enzyme should not be distorted too much or the effect will not occur, as illustrated (Table 3) by the enzyme form containing 3-propylPQQ which is not activated by Ca^{2+}, whereas this occurs in the one containing 3-methylPQQ (suggesting that the bulky propyl group prevents proper positioning, leading to suboptimal polarization). The fact that the enzyme form containing N1-methylPQQ showed reasonable activity is an argument to exclude mechanisms of sGDH (*26*) in which deprotonation at N1 (intramolecular general base catalysis) or formation of a *p*-iminoquinone structure is required.

All enzyme forms exhibited the negative cooperativity effect at about the same glucose concentration, implying that the switch in mechanism related to this phenomenon is not governed by the type of cofactor or activating metal ion present in the enzyme. No abnormalities were seen with the enzyme forms having low activity when studying their reductive half-reactions (in case the switch in redox state is the underlying cause, the slow change in redox state of the subunits occurring during the reduction of these enzyme forms would be easily seen as changes in the rate constant during the conversion). Therefore, the observations described here support an already proposed idea (*10*) that concomitant binding of two substrate molecules to the dimeric sGDH induces the negative cooperativity effect. Since the turnover number of Ba-E is so low that stopped-flow experiments can be performed, even in the high substrate concentration range, it becomes feasible now to investigate the reason for the change in mechanism.

Implications of the Catalytic Mechanism. The substantial KIE on the rate of fluorescent intermediate formation of Ba-E with glucose and that on the rate of its decay in Ba-E as well as in sGDH indicate that C−H bond breaking occurs in both processes. Since C−H bond breaking will not take place when PQQ in sGDH forms a hemiketal adduct with glucose, the fluorescing intermediate must be C5-reduced PQQ. The conclusion that the mechanism proceeds according to direct hydride transfer is supported by the fact that the adduct-forming ability of the cofactor in the enzyme is not related to its catalytic performance. Although in theory substrate oxidation could also proceed via sequential one-electron transfer steps, the absence of a detectable spectral intermediate resembling the semiquinone form of PQQ [similar to that occurring in MDH (*27*)] implies that if it occurs, it can experimentally not be distinguished from hydride transfer.

The large variation in specificity constant values of sGDH (*7*) and the large difference in rates of fluorescing intermediate formation from glucose and Ba-E (Table 2) could be due to either a large variation in substrate affinity for the enzyme (k_1/k_2 values) or a variation in hydride transfer rates (k_3 values). Since all the substrates have the same H/OH configuration at the site where the hydride is removed but differ from glucose only in having an opposite H/OH configuration at another site (this is at least the case for hexose sugars), the latter possibility seems unlikely. The availability of Ba-E, with which stopped-flow studies can be performed with undeuterated substrates, opens avenues now for further investigations on this aspect.

The proposed sequence of events and also several observations are not compatible with the previously proposed kinetic mechanism (Scheme 1), but they are with the new one (Scheme 2). In the latter, the enzyme and substrate form a complex (ES), after which deprotonation and hydride transfer take place, leading to formation of the fluorescing intermediate (E′P). Whether the transfer is reversible and whether the product is already released cannot be decided yet [release of product in the reductive half-reaction has been proven by product analysis (*28*) and is also suggested by the ping-pong mechanism in steady-state kinetics]. However, the fact that glucono-δ-lactone is not an inhibitor (*7*, *29*) suggests that either the reaction step is irreversible or it is reversible but the product is still attached to the enzyme (the latter possibility is depicted in Scheme 2). Subsequently, the fluorescing intermediate irreversibly tautomerizes to the reduced enzyme (E″).

When the kinetic mechanism is taken into account, the chemical mechanism must be as shown in Scheme 3. It is

known for several bivalent cations, including Ca^{2+}, that although they are biochemically redox inactive, they can nevertheless assist in enzymatic redox catalysis by activating the substrate. The role of Ca^{2+} as proposed here for the oxidoreductase sGDH is different from that as it concerns activation of a cofactor.

The structural information is completely in line with the chemical mechanism presented here. Studies on the complex of $sGDH_{red}$ with glucose revealed (*23*) that the β-anomer of glucose is situated more or less parallel to the plane of PQQ in such a way that the anomeric H, not the OH, is pointing (at a short distance) to the C5 of PQQ, the OH group interacting among others with His144. Thus, unless it is assumed that glucose becomes bound to $sGDH_{ox}$ in a different way or that the β-anomer turns into the α-anomer during the reaction (highly unlikely in view of the interactions of the OH group), these findings support the chemical mechanism proposed here. In view of the strong pH effect exerted on formation of the fluorescing intermediate formed in the reaction of Ba-E with glucose (Table 2), His144 could well be the base initiating hydride transfer from the substrate by accepting the proton from the OH group at the C1 position of glucose. It should also be noted that the structural information as well as the results presented here contradicts a mechanism in which the C4 carbonyl group functions as a hydride ion acceptor, as has been proposed by others (*26*).

Is the mechanism proposed here for sGDH also valid for other PQQ-containing dehydrogenases? When the enzyme most related to sGDH is considered with respect to the catalyzed reaction, the membrane-bound quinoprotein glucose dehydrogenase (mGDH), striking differences in properties can be identified. Although PQQ in mGDH is activated by Mg^{2+} instead of Ca^{2+}, we have shown that the quite different absorption spectra, compared to those of sGDH, must be due to a different mode of binding of PQQ and not to differences in the activating metal ion (*28*). Therefore, it seems unlikely that mGDH has the same mechanism as sGDH, but definite proof must await results of mechanistic studies on it.

Several years ago, we postulated that adduct formation between PQQ and methanol is involved in the mechanism of MDH (*27*). This was based on our findings that PQQ−alcohol adducts fluoresce (*16*), and that a fluorescing transient was observed when deuterated methanol was added to MDH_{ox}, the transient interpreted to be the PQQ−deuterated methanol adduct of the enzyme. This view was strengthened when we found that the mechanism-based inhibitor cyclopropanol forms a PQQ−3-propionaldehyde adduct at the C5 position with similar fluorescing properties (*17*). However, in the light of present knowledge, the fluorescing transient could also have been the C5-reduced PQQ in the enzyme. Despite the striking overall similarity of MDH and sGDH with respect to positioning of Ca^{2+} and PQQ, it should also be noted, however, that a number of differences exist which could result in different mechanisms: PQQ in MDH is in contact with the indole ring of a tryptophyl residue and has an unusual sulfur bridge (originating from two adjacent cysteinyl residues) in its vicinity, while sGDH has neither of them; MDH has an aspartyl residue in the putative active site which has been postulated to act as a general base catalyst, while sGDH does not have such a residue there but here His144 may play such a role; and MDH needs an activator (ammonia or an amine) and a high pH for activity, while sGDH does not. Moreover, the model studies on PQQ and methanol (*21*) are in line with a mechanism proceeding via a PQQ−methanol adduct, and recently, it has been reported that even structural evidence exists for this in MDH (*30*). However, just as for mGDH, from the lack of relevant kinetic data, we conclude it is too early to predict a mechanism for MDH and for the other types of PQQ-containing alcohol dehydrogenases.

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