On the Mechanism and Specificity of Soluble, Quinoprotein Glucose Dehydrogenase in the Oxidation of Aldose Sugars†

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ABSTRACT: Kinetic and optical studies were performed on the reductive half-reaction of soluble, quinoprotein glucose dehydrogenase (sGDH), i.e., on the conversion of $sGDH_{ox}$ plus aldose sugar into $sGDH_{red}$ plus corresponding aldonolactone. It appears that the nature and stereochemical configuration of the substituents at certain positions in the aldose molecule determine the substrate specificity pattern: absolute specificity exists with respect to the C_1-position (only sugars being oxidized which have the same configuration of the H/OH substituents at this site as the β-anomer of glucose, not those with the opposite one) and with respect to the overall conformation of the sugar molecule (sugars with a $^4C_1$ chair conformation are substrates, those with a $^1C_4$ one are not); the nature and configuration of the substituents at the 3-position are hardly relevant for activity, and an equatorial pyranose group at the 4-position exhibits only aspecific hindering of the binding of the aldose moiety of a disaccharide. The pH optimum determined for glucose oxidation appeared to be 7.0, implying that reoxidation of $sGDH_{red}$ is rate-limiting with those electron acceptors displaying a different value under steady-state conditions. The kinetic mechanism of sGDH consists of (a) step(s) in which a fluorescing intermediate is formed, and a subsequent, irreversible step, determining the overall rate of the reductive half-reaction. The consequences of this for the likeliness of chemical mechanisms where glucose is oxidized by covalent catalysis in which a C_5-adduct of glucose and PQQ are involved, or by hydride transfer from glucose to PQQ, followed by tautomerization of C_5-reduced PQQ to $PQQH_2$, are discussed. The negative cooperative behavior of sGDH seems to be due to substrate-occupation-dependent subunit interaction in the dimeric enzyme molecule, leading to a large increase of the turnover rate under saturating conditions.

Soluble, quinoprotein glucose dehydrogenase (EC 1.1.99.17; sGDH)[1] is a homodimeric enzyme which occurs in the bacterium *Acinetobacter calcoaceticus* (*1*) [quinoprotein, PQQ-containing; PQQ, pyrroloquinoline quinone (Figure 1A)]. The enzyme oxidizes a broad range of aldose sugars, the spectrum comprising hexoses as well as pentoses and mono- as well as disaccharides. The sugars are converted into their corresponding aldonolactones, and a variety of unnatural compounds such as *N*-methylphenazonium methyl sulfate (PMS) (*2*), 2,6-dichlorophenolindophenol (DCIP) (*3*), Wurster's Blue (*4*), N-substituted nitrosoanilines (unpublished results), and electroconducting polymers (*5*) act as electron acceptor for the enzyme in this. Structural aspects regarding the dimerization of the subunits, the binding of PQQ, and the role of Ca^{2+} and substitutes of it in this and in enzyme activation have already been described (*6*).

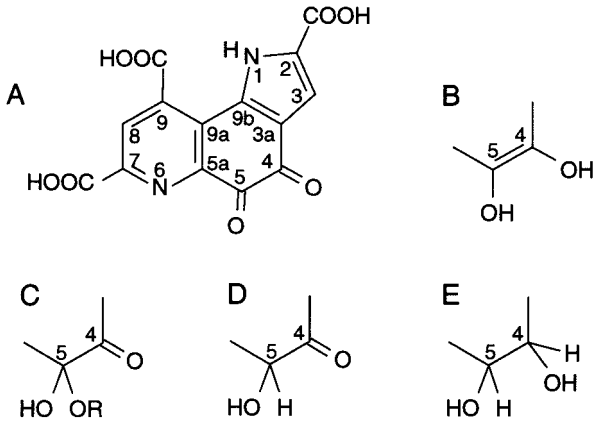

FIGURE 1: Structure of PQQ, some of its derivatives, and $PQQH_2$. (A) PQQ; (B) $PQQH_2$; (C) PQQ adducts (R=H, PQQ−H_2O adduct); (D) C_5-reduced PQQ; (E) $PQQH_4$.

Just like for other types of NAD(P)-independent, dye-linked dehydrogenases, the catalytic cycle of PQQ-containing dehydrogenases proceeds according to a ping-pong mechanism. So far, the best studied quinoprotein enzyme was methanol dehydrogenase (EC 1.1.99.8). However, although additional details of its kinetic mechanism (*7*) and the 3-D structure of its reduced form (*8*, *9*) are known, the chemical mechanism of alcohol oxidation by this enzyme is still obscure. Progress in this is hampered by the following difficulties: the instability of the oxidized form of methanol

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[1] Abbreviations: sGDH, soluble quinoprotein glucose dehydrogenase; PQQ, pyrroloquinoline quinone (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]-quinoline-4,5-dione); $PQQH_2$, quinol form of PQQ; $PQQH_4$, 4,5-dihydro-$PQQH_2$ (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dihydro-4,5-diol); PMS, *N*-methylphenazonium methyl sulfate; DCIP, 2,6-dichlorophenol indophenol; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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dehydrogenase (*10*); the presence of endogeneous substrate in enzyme preparations and of alcohols/aldehydes in buffer solutions (*11*); the still not understood role of NH_3 as activator in the catalytic mechanism of the enzyme (*1*). Since these difficulties are not met with sGDH, it seems an ideal model enzyme to study the mechanism of PQQ-containing dehydrogenases. In addition, since sGDH has large potentials for analytical applications (*5*, *12*), insight into its substrate and anomer selectivity is highly desirable.

The oxidized and the reduced enzyme forms (containing $PQQH_2$, pyrroloquinoline quinol, Figure 1B) of sGDH have been isolated (*2*), the properties and mechanism of formation being in line with a ping-pong mechanism for the enzyme. The results of steady-state kinetic investigations on sGDH supported this but also revealed some complicating factors: depending on the electron acceptor used, a different optimal pH was found (*2*, *4*); substrate inhibition occurs (*3*, *4*, *13*, *14*) (i.e., the sugar competes with electron acceptor for interaction with the reduced enzyme form); negative cooperative behavior is observed in the oxidation of sugars in the higher concentration ranges (*14*, *15*). To obtain more insight into this and to increase our knowledge on the mechanism and selectivity of sGDH, the first part of the catalytic cyle, i.e., the reductive half-reaction, was investigated. This was carried out in two ways: the stepwise reaction in which oxidized enzyme is titrated with sugar (anomers); the fast reaction in which oxidized enzyme is rapidly mixed with an excess of sugar. To detect any intermediate and to determine the reaction rates, changes in the status of PQQ were monitored in time with ultraviolet/visible and fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Apo- and Holo-sGDH Preparations. Apo-enzyme was isolated from an *Escherichia coli* recombinant strain containing the gene for sGDH from *Acinetobacter calcoaceticus* (*16*) as described (*2*). Holo-sGDH was prepared by adding excess PQQ to the apoenzyme, in the presence of Ca^{2+} ions, followed by gel filtration to remove unbound PQQ, as described (*6*).

Spectrophotometry. Ultraviolet/visible absorption spectra were measured with a Hewlett-Packard 8524A photodiode array spectrophotometer. When necessary, O_2 was removed from the samples by sparging with N_2. During the measurements and the addition of anaerobic glucose solution, the cuvettes were kept anaerobic by passing N_2 above the sample.

Steady-State Kinetics. Initial reaction rates were determined spectrophotometrically at 20 °C, as described previously (*2*), by monitoring the reduction of DCIP, using PMS as the primary electron acceptor. Based on the assumption that the observed negative cooperative behavior is due to subunit interaction (*15*), the dependence of the steady-state rate, v, on substrate concentration, [A], is described by

$$v = \frac{[A]^2 k_{cat2} + [A] k_{cat1} K_2}{[A]^2 + [A] K_2 + K_1 K_2} \quad (1)$$

in which k_{cat1} and K_1, and k_{cat2} and K_2 are sets of apparent kinetic parameters representing the enzyme's catalytic (Michaelis−Menten) performance at low and high sugar substrate concentration ranges, respectively (*17*, *18*). The specificity constant, defined as k_{cat}/K_M, was determined here from the initial slope in a plot of rate versus substrate concentrations (low range), represented by k_{cat1}/K_1.

Stopped-Flow Kinetics of the Reductive Half-Reaction. Stopped-flow experiments were performed by rapid mixing in a Hi-Tech Scientific SF53 apparatus and monitoring the absorbance at 337 nm (this wavelength giving the maximal difference in absorbance between oxidized and reduced sGDH). Experiments in which fluorescence or multiple wavelength absorbance changes were monitored were carried out with an Applied Photophysics SX.17MV stopped-flow reaction analyzer. All experiments were performed at 15 °C.

Mathematical Analysis of Stopped-Flow Traces. The reductive half-reaction was assumed to proceed according to the following scheme:

Scheme 1

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

where E and E′ represent the oxidized and reduced enzyme form, respectively. The formation of E′P from ES is considered to proceed in an irreversible way. This assumption is based on the large difference in redox potential between the couples glucose/glucono-δ-lactone [$E^{\circ\prime} = -364$ mV (*19*)] and PQQ/$PQQH_2$ [$E^{\circ\prime} = +50$ mV, in sGDH (*20*)] and the observation that addition of glucono-δ-lactone did not affect reaction rates in the steady-state assay. Provided that [S] is considerably larger than [E], the formation of E′P can be described by

$$\frac{[E'P]}{[E_{tot}]} = 1 - \frac{1}{k_\alpha - k_\beta}(k_\alpha e^{-k_\beta t} - k_\beta e^{-k_\alpha t}) \quad (2)$$

with k_α and k_β as the high and low observed rate constants, respectively, given by

$$k_\alpha = {}^1/_2(p + \sqrt{p^2 - 4q}) \quad (3a)$$

$$k_\beta = {}^1/_2(p - \sqrt{p^2 - 4q}) \quad (3b)$$

with $p = k_1[S] + k_2 + k_3$ and $q = k_1[S]k_3$ (*21*). Note that the relative amplitudes of the exponential terms are directly related to the observed rate constants and that apparent single-exponential E′P formation, with k_β as observed rate constant, will thus occur under conditions where $k_\beta \ll k_\alpha$ [which depends both on the relative values of the kinetic rate constants (e.g., when $k_2 > k_3$, i.e., rapid equilibrium of the first step) and on the substrate concentration used (e.g., when $k_1[S] \ll k_3$ or when $k_1[S] \gg k_3$)]. From the first derivative of eq 3b, it follows that the initial slope of k_β vs [S] equals the steady-state specificity constant, k_{cat}/K_M, since

$$\frac{dk_\beta}{d[S]}([S] = 0) = \frac{k_1 k_3}{k_2 + k_3} = \frac{k_{cat}}{K_M} \quad (4)$$

Fitting of Experimental Data. Computer fitting of experimental data was carried out with the Igor Pro v. 2.02 software package (WaveMetrics Inc., Lake Oswego, OR).

RESULTS

Anomer Preference of sGDH. Titration of holo-sGDH (in the oxidized form) with glucose under anaerobic conditions

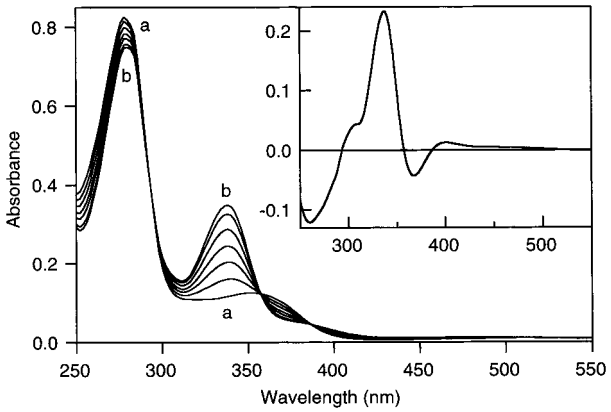

FIGURE 2: Absorption spectra taken during titration of sGDH with glucose. To sGDH (0.5 mg/mL) in 20 mM potassium phosphate buffer, pH 7.0 (spectrum a), were added 5 aliquots of glucose solution (prepared 1 day in advance as to be in anomeric equilibrium) with 30 s intervals, and spectra were taken between the steps. Finally excess glucose (90 μM final concentration) was added (spectrum b). The spectra were corrected for dilution (up to 3%) caused by the additions. The inset shows the difference spectrum obtained by subtraction of spectrum a from spectrum b.

resulted in gradual reduction of the enzyme without any indication for the existence of intermediates spectroscopically distinct from oxidized or reduced enzyme (Figure 2). Since glucose dissolved in water anomerizes to an equilibrium of its two anomeric forms and we wanted to establish the anomer specificity of sGDH, conversion experiments were carried out with fresh and equilibrated solutions of the two glucose anomers. Addition of a substoichiometric amount of a freshly (about 5 min earlier) prepared solution of either α-D-glucose or β-D-glucose resulted in immediate increases (i.e., within the time required to mix the sample) in absorbance at 338 nm of 8.7% and 149%, respectively, compared to that observed with the same amount of a solution of α-D-glucose which had been allowed to mutarotate to anomeric equilibrium for 1 day. Furthermore, with fresh α-D-glucose, the immediate absorbance increase was followed by a slow gradual absorbance increase, which can be attributed to mutarotation of the α- to the β-anomer. Since the immediate increase in absorbance observed with the fresh solution of α-D-glucose can be well accounted for by the expected amount of β-D-glucose formed by mutarotation within 5 min (*22*), it is concluded that sGDH only acts on the β-anomer of D-glucose. This is in agreement with experiments carried out with an amperometric sGDH enzyme electrode (A. J. J. Olsthoorn, unpublished results), also showing that an absolute preference exists for β-D-galactose, β-D-xylose, and α-L-arabinose (aldoses all having the same configuration at the anomeric C-atom as β-D-glucose). Based on an anomeric equilibrium composition for D-glucose of 37% α- and 63% β-D-pyranose (*22*), the following molar extinction coefficients for the catalytic center (=subunit) of sGDH were determined from the spectra shown in Figure 2: ϵ_{338} (difference of reduced minus oxidized enzyme) = 27.0 mM^{-1} cm^{-1}; ϵ_{280} (reduced form) = 87.0 mM^{-1} cm^{-1}; ϵ_{278} (oxidized form) = 95.9 mM^{-1} cm^{-1}. Using a value of 50 kDa for the subunit molecular mass (*16*), the following specific absorption coefficients were calculated: 1.74 L g^{-1} cm^{-1} at 280 nm for reduced sGDH; 1.92 L g^{-1} cm^{-1} at 278 nm for oxidized sGDH.

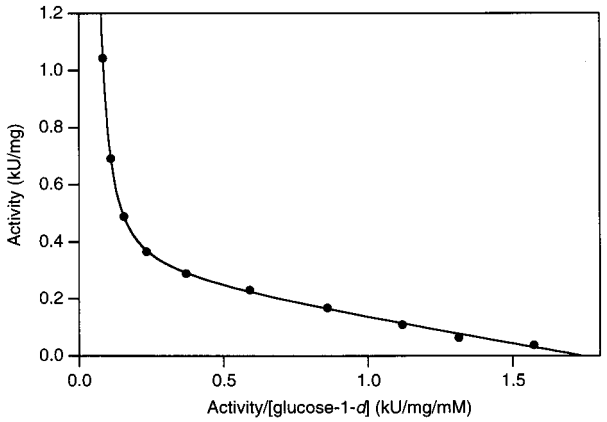

FIGURE 3: Eadie−Hofstee plot for sGDH with varying concentrations of glucose-1-*d*. Enzyme activities were measured as described under Experimental Procedures. The solid curve represents the fit of the experimental data with eq 1.

Table 1: Apparent Kinetic Parameter Values of sGDH for Glucose-1-*d* and for Glucose As Determined with Steady-State Kinetics[a]

	glucose-1-*d*	glucose[b]	k_H/k_D
k_{cat1} (s^{-1})	253 ± 4	1550 ± 190	6.1 ± 0.8
K_1 (μM)	171 ± 6	740 ± 180	4.3 ± 1.1
k_{cat2} (s^{-1})	4450 ± 610	17200 ± 1600	3.9 ± 0.6
K_2 (mM)	69 ± 12	69 ± 12	1.0 ± 0.3
K_I (mM)	−	92 ± 13	−

[a] Measurements were carried out as described in the legend of Figure 3, and kinetic parameter values ± SE were obtained by fitting the data in Figure 3 with eq 1. [b] Data taken from ref 15.

Steady-State Kinetics with Glucose-1-d. Analogous to previous results with glucose and other substrates (*15*), pronounced negative cooperative behavior was observed in the steady-state kinetics of sGDH with glucose-1-*d* (Figure 3). The experimental data could be adequately fitted with eq 1. The apparent kinetic parameter values obtained with glucose-1-*d* (Table 1) differ significantly from those found with nondeuterated glucose, resulting in substantial deuterium isotope effects, k_H/k_D. Substrate inhibition, occurring with glucose at high concentrations (*15*), has not been taken into account in fitting the data of glucose-1-*d*, as it was not observed in the range of substrate concentrations used here. However, since it cannot be excluded that it occurs, some caution applies to the reliability of the values obtained for k_{cat2} and K_2.

Stopped-Flow Studies of the Reductive Half-Reaction. Upon mixing sGDH with glucose-1-*d*, the absorbance at 337 nm increased in a non-single-exponential manner (Figure 4), but the trace could be adequately fitted with eq 2. The latter implies that the molar absorption coefficients of E and ES must be very similar at this wavelength. To obtain the individual rate constants indicated in Scheme 1, values of k_α and k_β were determined for several concentrations of glucose-1-*d* (Figure 5), and the data were simultaneously fitted to eq 3a for k_α, and to eq 3b for k_β, the fits yielding the parameter values given in Table 2.

Based on the reaction sequence shown in Scheme 1, a calculated absorption spectrum (Figure 6) of the intermediate species in this was obtained by analyzing the absorbance changes of sGDH in the range from 260 to 410 nm, caused by rapid mixing of the enzyme with glucose-1-*d* (0.1 mM),

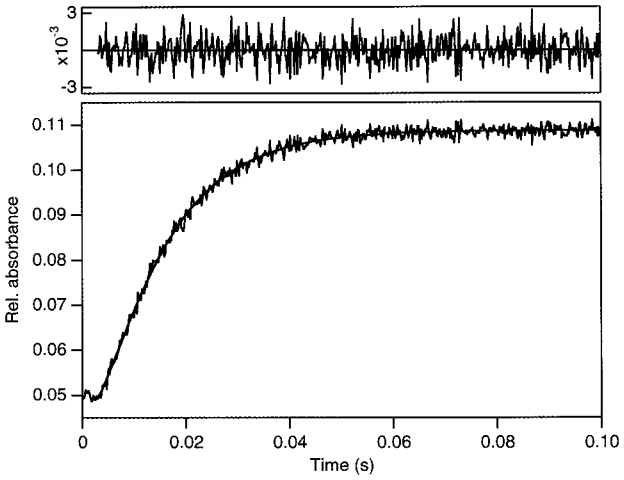

FIGURE 4: Reductive half-reaction of sGDH with glucose-1-*d*. Glucose-1-*d* (162 μM) was mixed in the stopped-flow apparatus with sGDH (9.6 μM subunit), in 50 mM potassium phosphate buffer, pH 7.0, and the absorbance at 337 nm was monitored in time. The smooth curve represents the fit of the data from 3.0 ms onward to eq 2, yielding values for k_α and k_β of 221 $\pm$ 13 and 83.3 $\pm$ 1.8 s^{-1}, respectively.

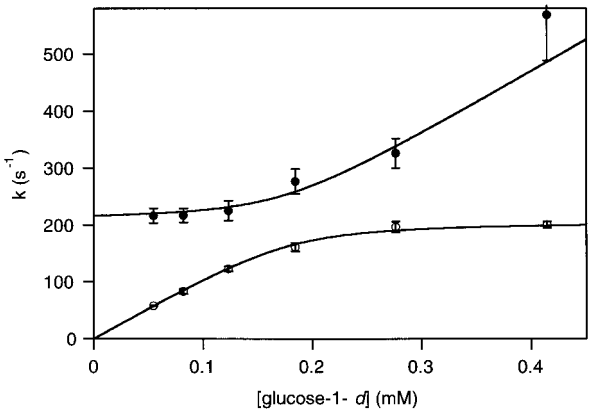

FIGURE 5: Plot of k_α and k_β versus glucose-1-*d* concentration. Values of k_α (closed circles) and k_β (open circles) were obtained by fitting the monitored absorbance change at 337 nm to eq 2. Results are means $\pm$ SE of three measurements. The solid curves represent the fitting of these values to eq 3a and eq 3b, respectively, yielding the rate constant values given in Table 2.

Table 2: Rate Constant Values of the Reductive Half-Reaction of sGDH with Glucose-1-d[a]

rate constant	from varying concentration	from multiple wavelength
k_1 (mM^{-1} s^{-1})	1129 $\pm$ 24	1136 $\pm$ 7
k_2 (s^{-1})	10.2 $\pm$ 2.4	fixed as 10.2
k_3 (s^{-1})	206.4 $\pm$ 4.4	218.4 $\pm$ 3.2

[a] Values $\pm$ SE were obtained as indicated in the legends of Figures 5 and 6.

with global analysis. As it is not possible to determine k_1, k_2, and k_3 independently at a single substrate concentration, in this case fitting was performed with a fixed value for k_2 of 10.2 s^{-1}. The values calculated for k_1 and k_3 with global analysis were close to those found for the single-wavelength experiments (Table 2). As illustrated by the spectra in Figure 6, this approach also shows that E and ES have similar absorbancies at 337 nm, justifying the use of eq 2 at this wavelength.

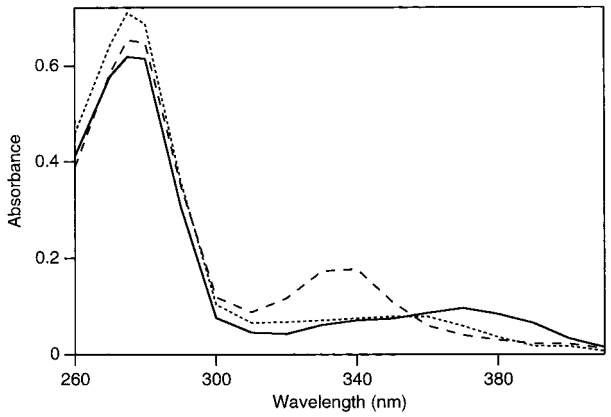

FIGURE 6: Calculated absorption spectrum of the reaction intermediate occurring in the reduction of sGDH with glucose-1-*d*. Experiments were performed as described in the legend of Figure 4, at a final glucose-1-*d* concentration of 0.10 mM. Absorbance changes were monitored at wavelengths of 260−410 nm in 10 nm steps and at 275 nm. The data were globally analyzed with Pro/Kineticist software (version 4.10, Applied Photophysics Ltd.), using the reaction model of Scheme 1 and a fixed value for k_2 of 10.2 s^{-1}. Spectra originate from the oxidized (···), intermediate (—), and reduced (- - -) forms of sGDH.

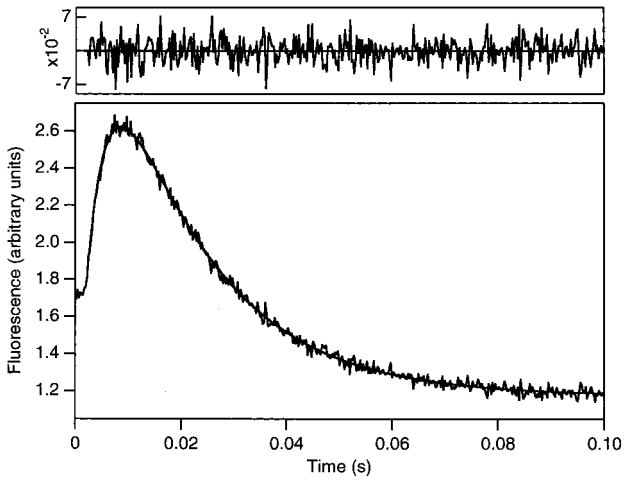

FIGURE 7: Transient fluorescence observed during reduction of sGDH by glucose-1-*d*. Glucose-1-*d* (0.10 mM) was mixed with sGDH (11 μM subunit) in 50 mM potassium phosphate buffer, pH 7.0. Excitation of the reaction mixture occurred at 380 nm, and the fluorescence emission was monitored only at λ >405 nm. The smooth line represents the fit of the data from 2.2 ms onward to a general double-exponential equation, yielding values for k_α and k_β of 247.7 $\pm$ 5.7 and 54.0 $\pm$ 0.6 s^{-1}, respectively.

On monitoring the fluorescence intensity when sGDH was mixed with glucose-1-*d*, a transient species was observed. The trace could be adequately fitted with a general double-exponential equation (Figure 7), which yielded values for the observed rate constants similar to those found from absorbance measurements (Figure 5). Therefore, this approach is also in accordance with the reaction sequence proposed in Scheme 1, with the fluorescing transient and the transient for which the absorption spectrum was calculated (Figure 6) being identical.

At higher concentrations of glucose-1-*d*, the observed rate constants (data not shown) became much higher than could be expected according to the mechanism and rate constant values deduced above for the low concentration range. This phenomenon is completely in line with the negative cooperative behavior observed in steady-state measurements, the

Table 3: Specificity Constant Values of sGDH for Various Aldoses[a]

	side group configuration[b]					k_{cat}/K_M[c] (mM^{-1} s^{-1})	
aldose	1 (%)	2	3	4	6	stopped-flow	steady-state[d]
D-glucose	63					1700	2100
1-deutero-						1080	1500
2-deoxy-	52.5	H				3.3	
3-deoxy-	55		H			2500	
6-deoxy-	64				H	390	
D-mannose	34.5	A				50	
D-allose	77.5		A			1470	
D-galactose	64			A		150	240
D-fucose	67			A	H	440	
D-xylose	63				X	46	45
D-lyxose	28	A			X	0.24	
D-ribose	58.5		A		X	11	
L-arabinose	60[e]			A	X	1450	
maltose				α-glc		800	
cellobiose				β-glc		1080	
lactose	63			β-gal		790	960
melibiose					α-gal	11	

[a] Values were calculated using eq 4 from observed rate constants for enzyme reduction obtained from stopped-flow measurements at three different substrate concentrations. [b] Schematic presentation of substrate structure as compared to β-D-glucose (Figure 8): %, percentage of β-D-pyranose anomer present at anomeric equilibrium (*22*, *34*); H, hydrogen instead of hydroxyl group (deoxyaldoses); A, configuration of hydroxyl group axial instead of equatorial; X, hydrogen instead of hydroxymethyl group (pentoses); glc, gal, D-gluco- or D-galactopyranosyl instead of hydroxyl group (disaccharides). [c] Apparent specificity constants based upon total substrate concentration, i.e., disregarding the existence of different (ratios of) anomeric forms. [d] Data taken from ref *15*. [e] α-Pyranose anomer; pentose nomenclature is based on the configuration at C_4, causing the pentose analogue of β-D-galactopyranose to be named α-L-arabinopyranose.

effect being characterized by a pronounced increase of the turnover number of the enzyme (*15*). Unfortunately, the observed rates exceeded the technical capabilities of the equipment, prohibitive for a proper interpretation and fitting of the traces.

Stopped-flow experiments with glucose (unlabeled) yielded apparent single-exponential increases in absorbance (traces not shown) throughout the range of substrate concentrations (0.05−0.3 mM) where measurable rates (i.e., with k_{obs} below 500 s^{-1}) were encountered. This behavior is consistent with the high value of k_{cat1} found with glucose in steady-state experiments (Table 1), which implies that k_3 is at least of a similar magnitude so that measurable rates can be obtained only under conditions where $k_1[S] \ll k_3$, resulting in apparent single-exponential behavior (see Experimental Procedures). Similar behavior (i.e., single-exponential traces and k_{obs} values surpassing the value of 500 s^{-1} with increasing substrate concentrations) was observed with a variety of other substrates, allowing determination of specificity constant values from k_{obs} values according to eq 4 (Table 3). With 2-deoxyglucose, fits of the traces (not shown) to a single exponential were not so perfect as for the other substrates. The reason for this deviation is unclear, but it is not caused by contamination of the 2-deoxyglucose preparation with another substrate because the phenomenon was observed for all concentrations tested (0.1−100 mM). No reduction of sGDH was observed with the aldoses D-arabinose, L-xylose, and L-rhamnose [having a $^1C_4$ chair conformation instead of the $^4C_1$ chair conformation as present in D-glucose (Figure 8)] and the D-glucose derivatives D-glucosamine and D-glucuronic acid.

Stopped-flow measurements of sGDH with glucose at different pH values showed that the reductive half-reaction has an optimum around pH 7.0 (Figure 9).

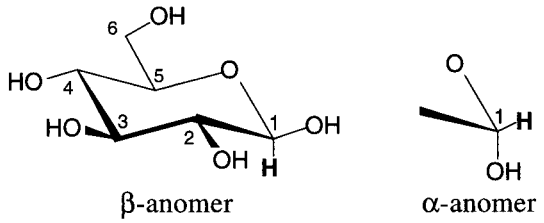

FIGURE 8: Stereochemical structure of D-glucose. The hydrogen which is deuterated in D-glucose-1-*d* is represented in boldface type.

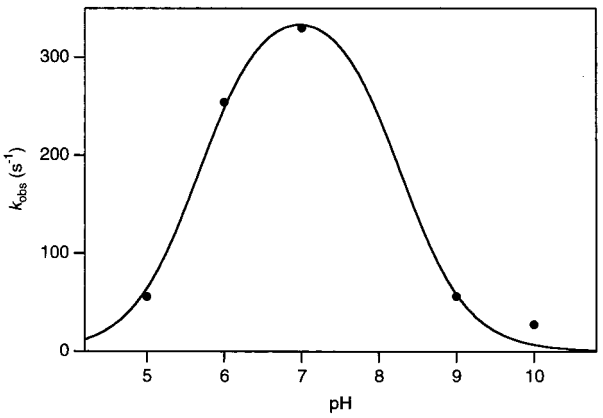

FIGURE 9: Dependence on the pH of k_{obs} for the reduction of sGDH with glucose. Experiments were performed as described in the legend of Figure 3, at a final glucose concentration of 0.20 mM, in 50 mM of the following buffers: citrate, pH 5.0; MES, pH 6.0; potassium phosphate, pH 7.0; CHES, pH 9.0; and CAPS, pH 10.0. The values of k_{obs} were calculated by fitting of the absorbance increase at 337 nm to a general single-exponential equation.

DISCUSSION

Specificity. The difference molar absorption coefficient for glucose-reduced minus oxidized enzyme at 338 nm of 27.0 mM^{-1} cm^{-1} corresponds fairly well with the value of 24.4 mM^{-1} cm^{-1} found by Hauge (*23*). However, in our case, the calculation is based on an absolute preference of

the enzyme for β-D-glucose whereas that of Hauge is not because he observed that all added glucose reacts with the enzyme. This suggests that under his conditions α-D-glucose mutarotated almost completely to the β-anomeric form in the time span between glucose addition and measurement of the resulting spectral change, although the cause of this rapid mutarotation is not clear and the phenomenon was not observed in our experiments.

Based on a previously obtained value for the specific absorption coefficient for reduced sGDH of 1.48 L g^{-1} cm^{-1} by ultraviolet/visible spectroscopy, it was calculated that holo-sGDH contains 2.3 Ca^{2+} ions per subunit (*6*). Recalculation, making use of the value for the specific absorption coefficient derived here (1.74 L g^{-1} cm^{-1}), yields 2.7 Ca^{2+} ions per subunit. Therefore, further research is required to establish the correct number of Ca^{2+} ions per subunit molecule in holo-sGDH.

In comparing the specificity constant data determined with steady-state and stopped-flow experiments (Table 3), it appears that both methods yield fairly similar values (note that the values obtained with the former method are nearly always somewhat higher than those obtained with the latter one, which is most probably due to the higher temperature at which the steady-state experiments were performed). Since the k_{cat1} (and thus k_3) values for the substrates tested were the same (*15*), differences in specificity constant values determined here must concern the performance of the substrate in the process leading to the fluorescent intermediate formation (related to k_1 and k_2). Thus, comparison of the specificity constants of the various substrates (Table 3) yields qualitative insight into the structural requirements posed by the active site of sGDH toward the substrate. The relatively low values observed with 2-deoxy-D-glucose, mannose, and lyxose and the high values found with 3-deoxy-D-glucose and allose indicate that the C_2-hydroxyl group of the substrate plays an important role in this process, whereas the C_3 substituents do not. The values for the disaccharides show that equatorial pyranosyl substituents at C_4 decrease the relative specificity by about a factor of 2, irrespective of the precise nature of the pyranose, suggesting that the decreased relative specificities are due to aspecific hindering of the interaction between the enzyme and the glucose moiety of the substrate. Interpretation of the results with other substrates differing from glucose with respect to their C_4 and/or C_5 substituents is less straightforward. The data seem to indicate a subtle interplay between C_4-hydroxyl and C_5-(hydroxy)methyl groups: an axial C_4-hydroxyl group can apparently compensate for a missing C_5-hydroxymethyl group (high value with arabinose compared to xylose) whereas the presence of both groups has an adverse effect (low value with galactose), and as suggested by the data for 6-deoxy-D-aldoses, the position of the C_4-hydroxyl group is irrelevant for the process [similar values for 6-deoxy-D-glucose and fucose (=6-deoxy-D-galactose)].

In assays for sGDH, the pH optimum appears to vary significantly with the artificial electron acceptor used, with reported values of pH 6 for DCIP (*3*), pH 7 for PMS/DCIP (*2*), and pH 9 for Wurster's Blue (*4*). This variation may be due to the cationic or anionic nature of the dye and to different amino acid residues involved in the reaction between dye and reduced enzyme, the residues exhibiting different pK_a values. The results reported here show that the reductive half-reaction exhibits an optimum around pH 7.0, indicating that reoxidation of reduced sGDH with electron acceptor is rate-limiting for DCIP and Wurster's Blue but not for PMS (the assay with the latter has a pH optimum of 7, and k_{cat1} and k_3 are similar).

Kinetic Mechanism. The similar values of k_{cat1} (Table 1) and k_3 (Table 2) for glucose-1-*d* indicate that the maximal attainable steady-state turnover rate (in the absence of the negative cooperativity effect) is mainly determined by the rate of the step in which reduced sGDH is formed (the small difference between the parameter values is probably due to the different temperatures at which they were determined). Thus, to explain the much higher value of k_{cat2}, it must be this step which is primarily affected by the mechanism underlying the cooperative behavior of sGDH. Whether this means just a removal of the barriers causing the rate-limitation or a complete change in mechanism remains to be elucidated.

The steady-state kinetic parameters obtained with glucose and glucose-1-*d* revealed a deuterium isotope effect of about 6 on k_{cat1}. In view of the reasoning given above, in fact the effect is exerted on k_3. Since hydrogen transfer reactions (which may concern a proton, a hydrogen radical, or a hydride ion) generally exhibit a primary isotope effect in the range of 6−10 (*24*), such a transfer may occur in the k_3-related reaction leading to reduced sGDH.

The isotope effect on the specificity constant for glucose can be used to establish whether isotope effects occur only in the step associated with k_3 or also in the previous steps connected with k_1 and k_2. When only k_3 shows an intrinsic isotope effect, the observed isotope effect on the specificity constant can be given (*25*) by

$$\frac{(k_{cat}/K_M)_H}{(k_{cat}/K_M)_D} = \frac{k_1 k_{3H}}{k_2 + k_{3H}} \frac{k_2 + k_{3D}}{k_1 k_{3D}} = \frac{1 + k_{3D}/k_2}{k_{3D}/k_{3H} + k_{3D}/k_2}$$

where the subscripts H and D denote parameters associated with normal and deuterated glucose, respectively. From the values of k_2 and k_3 found for glucose-1-*d*, it follows that the isotope effect on the specificity constant in this case could not exceed the value of 1.05 (obtained at infinite k_{3H}). The observed value is about 1.5 [1.4 from steady-state measurements, 1.6 from stopped-flow measurements (Table 3)], indicating the presence of an isotope effect on the step(s) associated with k_1 and k_2 as well. Whether it concerns a secondary isotope effect or a primary one on a step which is scarcely rate-limiting remains to be elucidated.

Regarding the mechanistic cause of the observed negative cooperativity, the results of the stopped-flow measurements indicate that it is not due to an enzyme preparation consisting of different sGDH species, as has also been concluded from steady-state measurements (*15*). Reduction of sGDH with glucose by titration did not reveal enzyme forms spectrally distinct from reduced or oxidized sGDH. This means that after one of the subunits becomes reduced, either it does not affect the absorption spectrum of the other, still oxidized subunit or the affinity of the latter for glucose is so increased that enzyme molecules in which only one of the two subunits is reduced do not occur. Both the latter explanation and the one proposing that the observed negative cooperativity is due to redox-state-dependent subunit interaction (i.e., reduction of a subunit affects the kinetic properties of the other

subunit) are unlikely since this would mean that the enzyme molecules do not behave uniformly during the course of the reaction, which was not observed in the stopped-flow measurements (at a low glucose concentration). It is suggested therefore that reduction of one of the subunits does not affect the optical or kinetic properties of the other, still oxidized subunit. This lack of communication between PQQ and $PQQH_2$ in the enzyme molecule also applies to electron transfer, as deduced from the fact that the semiquinone radical form of PQQ was not detected in half-reduced preparations with EPR spectroscopy. Since the kinetic performance of sGDH at low and high substrate concentrations is clearly different, as shown by the results of steady-state kinetics (*15*) and here with those of pre-steady-state kinetics of glucose-1-*d*, substrate-occupation-dependent subunit interaction (i.e., occupation of a subunit by substrate affects the kinetic behavior of the other subunit) is the most likely mechanism responsible for the observed negative cooperativity. Whether this concerns a real Michaelis−Menten complex of oxidized enzyme subunits with substrate or a situation in which one of the subunits has already reacted to an intermediate stage (e.g., the fluorescing intermediate) is not clear. However, the first possibility seems more likely since the second possibility would probably have been detected in the stopped-flow experiments with glucose-1-*d*.

Chemical Mechanism. PQQ reacts with a large number of nucleophiles, including alcohols and aldehydes, to a fluorescing adduct at the 5-position (*26*) (Figure 1C). Thus, when a fluorescing enzyme species was detected in the reaction of methanol dehydrogenase with (deuterated) methanol, a covalent catalysis mechanism in which this adduct plays a central role was proposed for the enzyme (*7*) [see a recent publication for possibilities to explain the chemistry leading to product formation from the adduct (*27*)]. The results of studies on the model reaction between a PQQ ester, methanol, and Ca^{2+} in an organic solvent support this view, one of the roles of the bivalent cation being ascribed to assisting in adduct formation (*28*). The results reported here for sGDH are compatible with such a mechanism: (a) reversible step(s) leading to the formation of a fluorescing intermediate in the catalytic cycle, having an absorption spectrum (Figure 6) compatible with that of PQQ adducts (*26*), the steps being rather insensitive to the deuterium isotope effect exerted by glucose-1-*d*; a subsequent, irreversible step in which the transfer of reducing equivalents takes place, being prone to the deuterium isotope effect. Also other properties are in line with this: the very low turnover number for glucose of Holo-X, an enzyme form of sGDH in which the "assisting Ca^{2+} ions" are lacking (*6*), but for which no fluorescing intermediate is observed in the catalytic cycle (A. Dewanti, unpublished results), indicating that the low turnover of this enzyme form could be due to the rate-limiting step of adduct formation between PQQ and substrate; the insensitivity of the maximal turnover rate and the UV−Vis spectrum of sGDH (oxidized or reduced) to substitution of Ca^{2+} by Sr^{2+}, Cd^{2+}, or Mn^{2+} (*6*), indicating that although PQQ is a ligand for the metal ions, the variation in properties of the metal ions does not affect the properties of PQQ in sGDH.

Although the covalent catalysis mechanism is generally accepted, the crucial evidence for it is still lacking: structural data for a PQQ−substrate adduct in the dehydrogenases are unavailable (the only indication in favor for this is the similarity of the fluorescence spectra of the intermediate with those of the genuine adducts formed between PQQ and a nucleophile in solution); it is unknown in which step product release takes place. In view of the uncertainty, could an alternative chemical mechanism be compatible with the kinetic mechanism?

Based on stereoelectronic considerations, the anomer specificity of aldose oxidoreductases has been predicted (*29*). Assuming that hydride removal from the C_1-atom of an aldopyranose takes place, the theory predicts that an optimally efficient catalyst should have an absolute specificity for the β-anomer of glucose, as is the case for NAD-dependent glucose dehydrogenase (*30*) and glucose oxidase (*31*). Since it appears now that sGDH is no exception to this rule, the question can be posed whether the aforementioned assumption also applies to this enzyme. It is generally accepted that oxidation of a CH(OH) moiety in a substrate by NAD(P)-dependent dehydrogenases proceeds via hydride transfer from the C-atom to the electrophilic site in the coenzyme, i.e., the 4-position in NAD(P). In one of the hypotheses, the catalytic Zn^{2+} is presumed to facilitate deprotonation of the OH group, enabling hydride transfer to take place. Extrapolating such a mechanism to quinoprotein dehydrogenases, Ca^{2+} could fulfill the assisting role (either by deprotonation of the OH at the 1-position or by polarizing the oxygen of the C_5-carbonyl group) so that hydride transfer can occur to the most electrophilic site in PQQ, i.e., the C_5-atom [without bringing this in line with experimental results, such a step has already been put forward by Anthony et al. (*32*) as a possible mechanism for methanol dehydrogenase]. Applying this to sGDH, the C_5-reduced PQQ (Figure 1D) should be equivalent to the observed fluorescing intermediate species, and the rate-limiting step should concern irreversible tautomerization of the C_5-reduced PQQ to the energetically more favorable reduced form, $PQQH_2$ (in view of the structural resemblance to the PQQ−H_2O adduct (Figure 1C (R=H)) and 4,5-dihydro-$PQQH_2$ ($PQQH_4$) (Figure 1E), the similarity of the fluorescence spectra of C_5-reduced PQQ to those of PQQ adducts is not unlikely; to explain the formation of $PQQH_4$ upon reducing PQQ with $NaBH_4$, the assumption that C_5-reduced PQQ exists is also required (*33*)]. Since the rate-limiting step in the hydride transfer mechanism does not concern the actual oxidation of substrate but the tautomerization of C_5-reduced PQQ to $PQQH_2$, this mechanism can easily explain why the maximal oxidation rates for the sugars are the same. In the covalent catalysis mechanism, including the one proposed in which the hydride is transferred to the oxygen of the C_4-carbonyl group (*27*), it has to be assumed that the nature of the sugar in the adduct complex is irrelevant for the rate-limiting step in the internal redox chemistry with which the complex is decomposed into aldonolactone and $PQQH_2$. On the other hand, in the hydride transfer mechanism, the low deuterium isotope effect exerted on the process in which actual hydride removal from the substrate takes place is somewhat curious. To explain this, it has to be assumed that hydride removal is not rate-limiting but another step in this process is. Summarizing, the kinetic mechanism as reported here for sGDH is compatible with both chemical mechanisms discussed above, and no strong arguments in favor of one of them can be put forward at the moment.

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