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Kinetic studies of the active sites functioning in the quinohemoprotein fructose dehydrogenase

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Steady-state kinetic analysis was performed on the reaction between D-fructose and ferricyanide with the quinohemoprotein fructose dehydrogenase from *Gluconobacter* species. The D-fructose oxidation dependence on the ferricyanide concentration resulted in a series of parallel reciprocal plots, and the reaction was assumed to proceed by a ping-pong type of mechanism. A reciprocal plot of the reduction of ferricyanide at saturating concentration of D-fructose gave a break which was considered to appear as a result of the two active centers, namely PQQ and heme c functioning. A scheme of action is proposed and the bimolecular rate constant of the D-fructose oxidation, the k_{cat} for PQQ and the electron transfer rate between the $PQQH_2$ and heme *c* are calculated and account for $2.2 \pm 0.4 \cdot 10^4$ M^{-1} s^{-1}, (93 ± 14) and (162 ± 7) s^{-1}, respectively.

D-Fructose dehydrogenase; Quinohemoprotein; Ferricyanide; Kinetics; Steady state

1. INTRODUCTION

During the past couple of years much attention has been paid to coupling of enzymatic reactions to electron transfer reactions at electrodes for selective and sensitive detection of a wide variety of compounds. A rather newly recognized class of enzymes, the quinoproteins [1–3], seems to fulfill many of the necessary criteria for incorporation into practical in vivo and in vitro biosensors [4,5] as they do not require additional soluble cofactors and do not suffer from oxygen interference. Hence studies on the PQQ-containing proteins have become of increasing interest.

Most of the bacterial quinoproteins are dehydrogenases, and, depending on the enzyme, the natural electron acceptor is either a *c*-type cytochrome, a 'blue' type I copper protein, or a membrane-bound ubiquinone [6]. Some of the above mentioned enzymes appeared to contain more than one cofactor in their active center. Methanol dehydrogenase from *Hypomicrobium X* has thus been reported to contain two PQQs, one of which is in the half-oxidized PQQH· state, which is unable to convert a substrate to product unless an activator is available [7]. The apoenzyme of the alcohol dehydrogenase from *Pseudomonas testosteroni* was found to be a monomer with an absorption spectrum similar to that of oxidized cytochrome *c*. After the reconstitution of the holoenzyme by addition of PQQ, addition of substrate changed the absorption spectrum to that of reduced cytochrome *c*, indicating that the heme *c* group participated in the enzymatic mechanism [1].

The best investigated PQQ-containing enzyme is methylamine dehydrogenase from *Paracoccus denitrificans*. The redox properties [8], the steady-state kinetic parameters of the reaction with physiological [9] as well as with non-physiological electron acceptors [10], and the complex formation between the enzyme and amicyanin [11] were reported.

D-Fructose dehydrogenase from *Gluconobacter industrius* was first detected by Yamada et al. [12]. Later on, Ameyama et al. [13] succeeded in purifying the enzyme to homogeneity and it was found to consist of three units, one of which was considered to be a heme of type *c*. A number of authors have reported on the application of the D-fructose dehydrogenase in biosensors operating for the quantitative determination of D-fructose [14–16], yet many of the data on the mechanism of the enzyme functioning are still lacking.

In this report we present a model for the electron transfer between the substrate, the two active sites of the enzyme, and the non-physiological electron acceptor ferricyanide.

2. MATERIALS AND METHODS

D-Fructose dehydrogenase, FDH, (D-fructose:[acceptor]5-oxidoreductase; EC 1.1.99.11) from *Gluconobacter* spp. was obtained as a lyophilized powder (Sigma, 31 U/mg solid) and used without further purification. The concentration of FDH was determined spectrophotometrically with the use of $\varepsilon_{550} = 23$ $mM^{-1} \cdot cm^{-1}$ [1]. D-Fructose (Serva) and potassium ferricyanide (Merck) were of research grade. The reaction rate was determined spectrophotometrically using a Perkin-Elmer Lambda 3 spectrophotometer using the decrease in fer-

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ricyanide absorption (ε_{420} = 1.02 $mM^{-1} \cdot cm^{-1}$). All the solutions were prepared using 0.1 M phosphate buffer, containing 0.1% Triton X-100, pH 4.5. Spectrophotometric titration with the substrate was performed by adding appropriate amounts of substrate to the sample as well as to the reference cell.

3. RESULTS AND DISCUSSION

Reciprocal plots, obtained when initial rates of the fructose oxidation were determined at different concentrations of fructose in the presence of a series of fixed concentrations of ferricyanide, exhibited a series of parallel lines (Fig. 1). When the initial rate was determined at different concentrations of ferricyanide in the presence of the fixed concentrations of fructose, a break was observed in the Lineweaver–Burk plots (Fig. 2). Previous studies on the kinetics of membrane enzyme complexes have proved that this behaviour is observed when several active sites are supposed to participate in the electron transfer. Flavoprotein NADPH:adrenodoxin reductase, functioning in the mitochondrial electron transport chain for the cytochrome P-450-dependent steroid hydroxylation, was thus found to give a break in the Lineweaver–Burke plot for the quinone reduction in the presence of adrenodoxin. This biphasicity was assumed to be a result of functioning either of adrenodoxinreductase or of its complex with adrenodoxin [17]. Analogous breaks are also characteristic of yeast flavocytochrome b_2, where quinoidal acceptors oxidize the heme at a higher rate constant than $FMNH_2$, but the electron transfer from $FMNH_2$ to heme is slower than the FMN reduction by lactate [18].

The absorption spectra in the visible region suggested

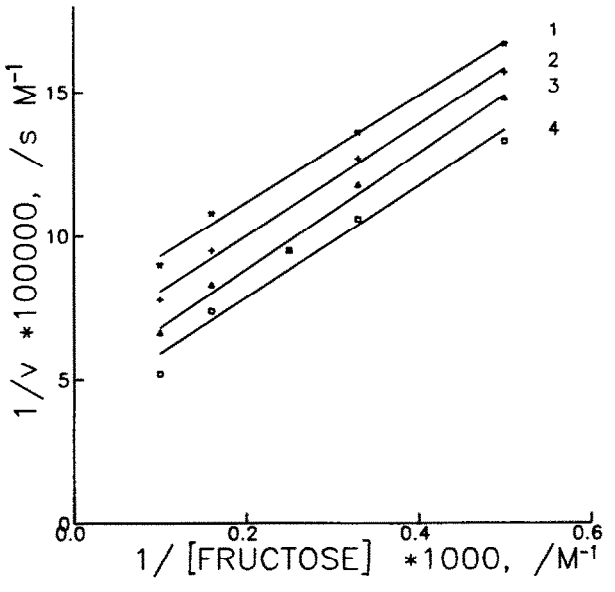


Fig. 1. The substrate dependence of the initial rate of the FDH-catalyzed oxidation of fructose by ferricyanide. Ferricyanide concentrations: 0.2 (1), 0.39 (2), 0.58 (3), 1 (4) mM. The enzyme concentration was 22.5 nM.

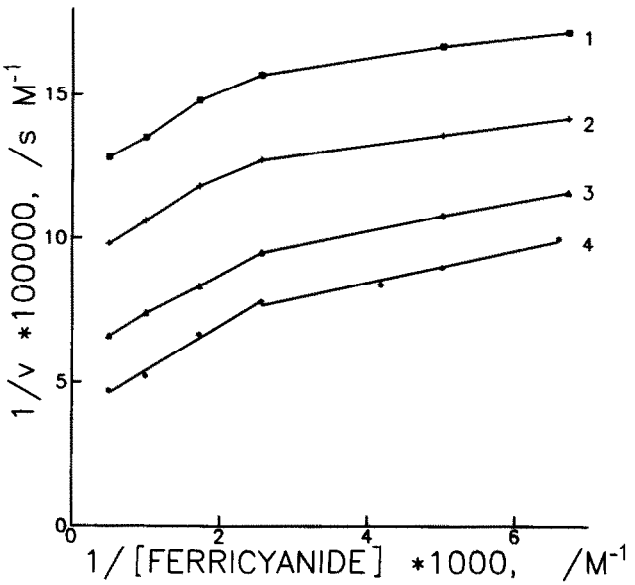


Fig. 2. The substrate dependence of the initial rate of the FDH-catalyzed reduction of ferricyanide by fructose. Fructose concentrations: 2 (1), 3 (2), 6 (3), 10 (4) mM. FDH = 22.5 nM.

that the enzyme, in addition to the PQQ, contains a hemoprotein with absorption maxima at 550, 523 and 417 nm (Fig. 3). These peaks are typical for the reduced heme *c*. The addition of fructose up to a concentration of 1 mM (the enzyme concentration was 1.13 μM) did not cause any changes in the heme *c* spectrum, suggesting that until the ferrocytochrome is re-oxidized by the electron acceptor, it does not participate in the catalytical processes. The experiments on the re-oxidation of the heme *c* were monitored while titrating the enzyme

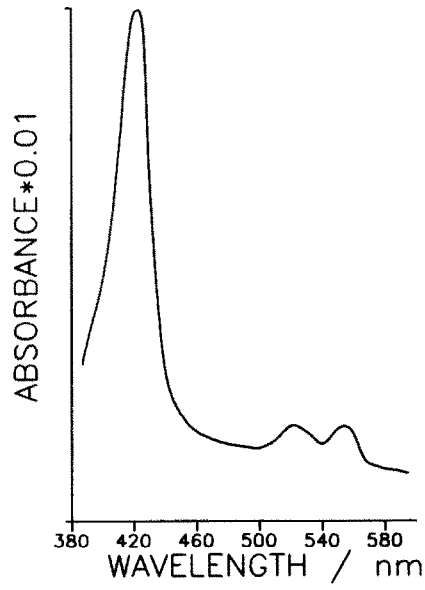


Fig. 3. The absorption spectrum of the heme part of the FDH.

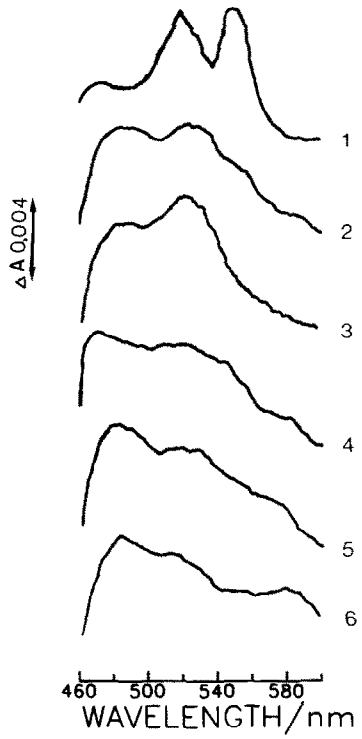


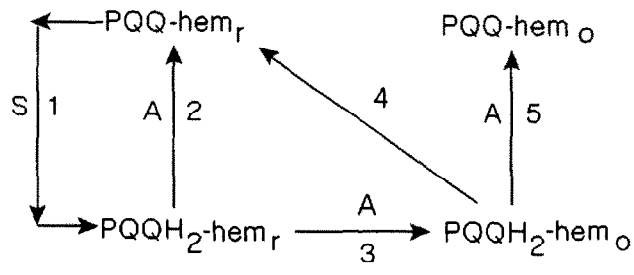
Fig. 4. Spectrophotometric titration of the heme *c* of the FDH with ferricyanide. Ferricyanide concentrations: 0–0.3 (1), 0.4 (2), 0.5 (3), 0.6 (4), 0.7 (5), 0.8 (6) mM. FDH = 740 nM.

with the ferricyanide in the concentration range of 0.05–0.8 mM. The results indicate either that ferricyanide up to the concentration of the 0.3 mM is unable to re-oxidize the heme *c*, or that the re-oxidation is so slow, that it can not be noticed within the time of the spectral scan (about 1 min). Addition of more than 0.4 mM ferricyanide resulted in the disappearance of the peak at 550 nm and gradual formation of the new peaks: one at 530, which could be specified as that of the oxidized heme *c*, and one at 490 nm, which cannot be identified at present (Fig. 4). It is reasonable to assume either that there are some steric hindrances for the access of ferricyanide to heme *c*, or that oxidation of the hemoprotein requires a rather high potential, resulting in a very slow re-oxidation at low concentrations of ferricyanide. That should be in accordance with the results of Khan et al. [16], who found the activity of fructose dehydrogenase, adsorbed on the Pt electrode, to be dependent on the adsorption potential. Maximum activities were obtained when the electrode potential was in the range of 0–0.1 V (the range of the PQQ redox potential), and also around 0.5 V, which could be the redox potential of heme *c*.

On the basis of these results we assume the catalytic process to occur according to Scheme 1, where S denotes fructose, and A ferricyanide.

The PQQ of the enzyme is reduced by fructose (pathway 1) and it can be re-oxidized already at low concentrations of ferricyanide (pathway 2). Nevertheless, when higher concentrations of ferricyanide are used, heme of the enzyme is re-oxidized (pathway 3) and the holoenzyme can be reconverted to its native redox state during the charge transfer between the reduced PQQ and the oxidized heme (pathway 4). Pathway 5, where the enzyme $PQQH_2$–$heme_o$ is oxidized to the PQQ–$heme_o$, is not excluded either. It should lead to a dead-end complex formation, as the totally oxidized enzyme was observed to dissociate and become inactivated, as in the case of methanol dehydrogenase, which was also isolated in a one-electron reduced form. The three redox forms were found in the catalytic cycle of this enzyme, and when fully oxidized the enzyme appeared to be inactivated [7].

Some kinetic parameters for the interaction of the fructose dehydrogenase with fructose, as well as with ferricyanide, were derived from secondary plots of the intercepts, plotted against substrate concentrations. Fig. 5A shows that intercepts, plotted for both phases, the 'slow' (at low ferricyanide concentrations) and the 'fast' one (at high ferricyanide concentrations), gave two parallel plots, which indicates that the bimolecular rate constants k_{cat}/K_M of the fructose oxidation are the same for both phases and account for $2.2 \pm 0.4 \cdot 10^4$ $M^{-1} \cdot s^{-1}$, while the k_{cat} varies. This provides evidence that the turnover of PQQ depends on the redox state of the holoenzyme, similarly as in the case of methylamine dehydrogenase, where its complex with the oxidized amicyanine appears to render the enzyme a more efficient catalyst [9]. The K_M for fructose, calculated from the fast phase, accounted for 11.8 ± 2.5 mM and is in good agreement with that reported by other authors [13]. Fig. 5B shows the intercepts from Fig. 1. The slow phase might represent the reaction of $PQQH_2$ with ferri-



Scheme 1

Table I

Steady-state kinetic parameters for the FDH in the fructose oxidation with ferricyanide

Phase	Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)
Slow	Fructose	95 ± 16	4.2 ± 0.8	$2.2 \pm 0.4 \times 10^4$
Slow	Ferricyanide	93 ± 14	0.011 ± 0.002	$8.2 \pm 1.0 \times 10^5$
Fast	Fructose	250 ± 56	11.8 ± 2.5	$2.2 \pm 0.4 \times 10^4$
Fast	Ferricyanide	162 ± 7	0.47 ± 0.06	$3.4 \pm 0.4 \times 10^5$

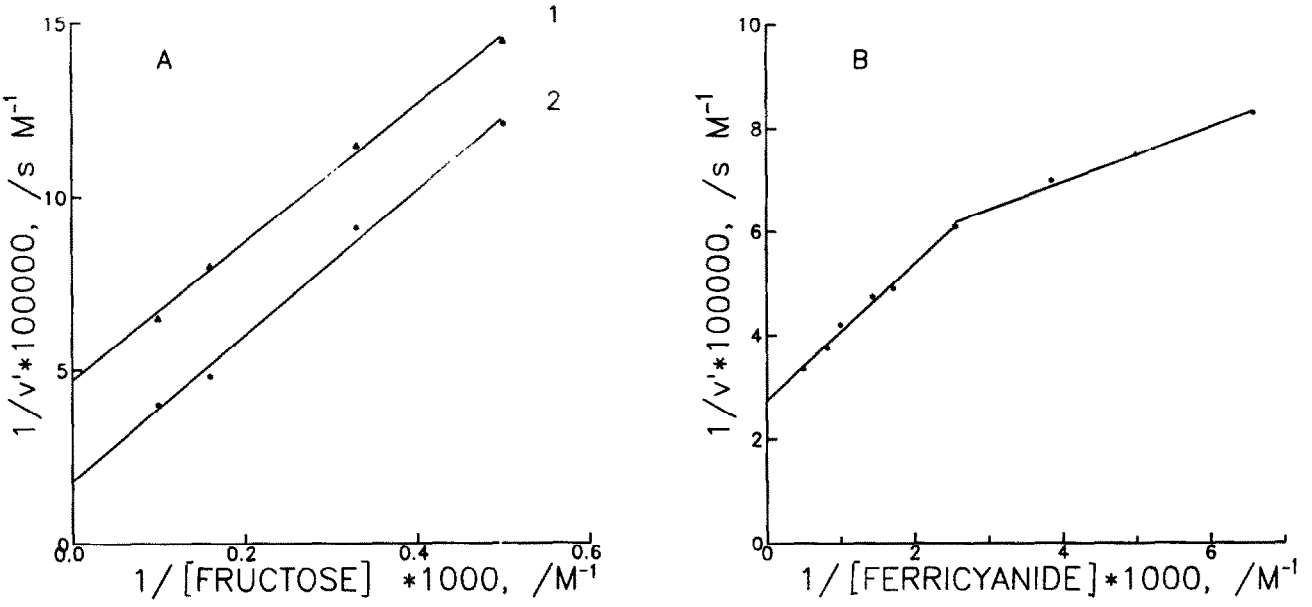


Fig. 5. Double-reciprocal plots of (A) fructose oxidation catalyzed by FDH (trace 1, slow phase; trace 2, fast phase), and (B) ferricyanide reduction, catalyzed by FDH.

cyanide. In this case, K_M of the ferricyanide for the $PQQH_2$ was 11 ± 2 μM, the k_{cat} 92.5 ± 13.5 s^{-1} and the bimolecular rate constant 8.2 ± 1.0·1.0^5 $M^{-1}·s^{-1}$. At higher concentrations of ferricyanide the kinetic constants gave quantitative information on the active holoenzyme functioning. The k_{cat}/K_M is lower, while the k_{cat} and K_M are higher (Table I).

The data presented above provide useful information concerning the use of PQQ-containing proteins capable of performing long-range intermolecular electron transfer in analytical systems.

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