PRODUCT BINDING AS MODULATOR OF FLAVIN REDOX PARAMETERS : A MECHANISM OF ACTIVITY CONTROL IN DEHYDROGENASE-e^-TRANSFERASE ?

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Introduction

Yeast L-lactate cytochrome c oxidoreductase or flavocytochrome b_2 is a complex two-domain enzyme that pertains, in the classification of flavoproteins defined by Hemmerich and Massey (1), to the class 4 (de)hydrogenase-e^-transferase. Indeed, the two electron-equivalents donated by lactate (yielding pyruvate), firstly accepted by flavin, are delivered one by one to the cytochrome moiety (preferential acceptor) with obligate formation of the semiquinone form, and hence to external acceptors (cf review (2)).

Equilibria concerning flavin-heme electron exchanges in flavocytochrome b_2 (from baker's yeast and Hansenula anomala) have already been thoroughly studied under conditions where no specific ligand other than the titrating reactant, ie L-lactate, was added to the system. The results (Blandin-Capeillère et al.(3)) are presented in Table 1.

New redox determinations have been more recently carried out with Hansenula anomala flavocytochrome b_2 in the presence of pyruvate since this reaction product was suspected to modify flavin-heme redox equilibria (4). Correlations were established between the intensity of EPR semiquinone signal observed at the titration temperature and the level of heme reduction deduced from the absorbance level at 556 nm. It was thus shown that pyruvate considerably increases the proportion of semiquinone which reaches 90±10% during titrations (5,6): midpoint potential of the F_{ox}/F_{sq} couple was shown to be markedly altered (by +60 mV) and the flavin dismutation constant increased by a factor x 20 , as shown in table 1.

The present potentiometric/spectrophotometric study confirms the drastic modifications of flavin redox behavior in the presence of pyruvate.

Flavins and Flavoproteins

Results

The reductive anaerobic potentiometric titration of flavin and cytochrome moieties of flavocytochrome b_2 presents the profile shown in fig 1 when pyruvate is present at 10 mM. Midpoint potentials are respectively found equal to -25 mV for the cytochrome, +55mV for F_{ox}/F_{sq}, and -137mV for F_{sq}/F_{hq} . These direct potentiometric values confirm the order of magnitude of earlier estimates (cf table 1). As shown in fig 2, flavin spectra, do not vary in the whole interval 0 to -80 mV corresponding to the level of heme reduction between 35 and 80 % and are typical of red semiquinones as already underlined (5,6). ε values for bound flavin under all redox states are established here with uncertainty of ± 1 $mM^{-1}cm^{-1}$) as previously discussed (6). Best estimates are $\varepsilon_{490-570} = 4.6$ $mM^{-1}cm^{-1}$ for the semiquinone and $\varepsilon_{460} = 10$ $mM^{-1}cm^{-1}$ for the oxidized form, both in the presence of pyruvate.

Table 1 : Midpoint potentials (mV) of heme and flavin of H-flavocytochrome b_2 at pH 7.00

[Pyruvate] ↓	t	$H_{ox/red}$	$F_{ox/sq}$	$F_{sq/hq}$	% F_{sq}	K_{dism} *1	Methods *2	Ref
Without	10°C	-25	-18	-42	40		P,S,E	*3
	18°C	(-25)	-20	-76	55	9	S,E	(5,6)
With 1 mM	"	(-25)	+14	-112	90	170	"	"
10 mM	"	(-25)	+35	-95	90	160	"	"
10 mM	"	-25	+55	-137	94	1000	P,E	this study

*1 : $K_{dism} = (F_{sq})^2/(F_{ox})x(F_{hq})$

*2 : Symbols : P for potentiometry, E for EPR estimate of F_{sq}, S for spectral study of F/H equilibria ; values for F are calculated from value for H in brackets.

*3 : Capeillère-Blandin & Bray : revised value (article in preparation).

Conclusions

Data in table 1 demonstrate that binding of the reaction product to the semiquinone form of such a dehydrogenase-e^-transferase, probably directly on the flavin (7), provokes a marked thermodynamic stabilization of the semiquinone, as shown by the K_{dism} value increasing by a factor of 20-100.

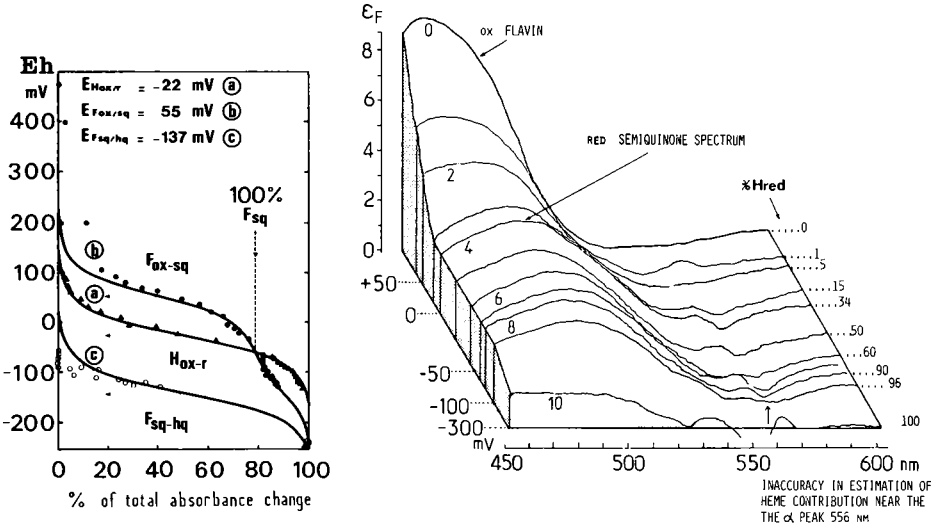

Fig 1: Spectrophotometric/potentiometric redox titration of flavin and cytochrome groups of flavocytochrome b_2 in the presence of 10 mM pyruvate (18°C).

Anaerobic redox titrations of flavocytochrome b_2 (35 µM in 100 mM phosphate buffer containing 10 mM pyruvate,final pH 7.00) are carried out, according to classical procedure, with the following mediators : Phenazine methosulfate: 3µM, Methylene blue: 1.4µM, Resorufine: 2µM, Naphtoquinone: 3µM. After an initial addition of ferricyanide (10 µM final), titration is carried out by addition of successive aliquots of L-lactate, each yielding a concentration increment of 2.5 µM, up to a total of 45 µM. Titration is completed by addition of aliquots of thiosulfite. Potentials and absorbance measurements at 6 wavelengths (λ-programm)(630,575,556,540,490,460nm) are performed after equilibration (1 to 4min). At critical phases during the titration, the whole spectrum is recorded and memorized. Flavin spectral contribution is calculated by substraction of heme contribution at each reduction level. Abscissa are for the different curves: a) % of absorbance variations at 556 nm, ie heme reduction level. b) % of flavin absorbance contribution at 460 nm ; 80% of the total variation corresponds to the F_{ox}/F_{sq} titration. c) % of flavin absorbance contribution at 490nm, near the isosbestic point of F_{ox}/F_{sq}. The curve shows therefore the titration F_{sq}/F_{hq} completed with thiosulfite. Solid lines are simulations based on Nernst formulation and values in table 1.

Fig 2: Evolution of flavin spectra during flavocytochrome b_2 anaerobic titration in the presence of 10 mM pyruvate.

Flavin spectra are computed from memorized partially reduced flavocytochrome b_2 spectra as described in (6). Baselines are taken as zero at 540 nm. Methylene blue contribution is visible at high E_h values between 550 and 600 nm. Intermediate spectra (35-90 % H_{red}) correspond to a red F_{sq}.

Since results at 1 and 10 mM pyruvate are not significantly different, the affinity of pyruvate for the flavosemiquinone enzyme must be higher than for the oxidized form (K_d = 5 mM)(unpublished) and similar to that of the substrate for the oxidized enzyme (K_m = 0.9 mM) .

The non-competitive inhibition exerted by pyruvate could be explained by the accumulation of an abortive flavosemiquinone-enzyme-pyruvate complex ; pyruvate, in this hypothesis, binds to an intermediate form of the enzyme, not to a site different from the substrate one.

This kind of thermodynamic control of the intermediate radical form and more generally of redox parameters governing the relative amounts of the three flavin species, in the presence of products at low and physiological concentrations, could be much more general and play an essential role in the retrocontrol of enzyme activity by reaction products.

Indeed, such an alteration of flavin redox parameters has been described by Gutman et al (8) for succinodehydrogenase in the presence of oxalacetate (not the direct product but a further keto-product in the citric cycle) and by Iyanagi et al (9) for NADH-cytochrome b_5 reductase in the presence of excess NAD^+.

References

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