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MODIFICATIONS OF REDOX EQUILIBRIA WITH SEMIQUINONE STABILIZATION UPON PYRUVATE BINDING TO L-LACTATE CYTOCHROME c OXIDOREDUCTASE (FLAVOCYTOCHROME b_2)

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SUMMARY : Spectral redox titrations of flavin and cytochrome b_2 moieties of flavocytochrome b_2 were achieved in the absence and in the presence of pyruvate under equilibrium conditions at 18° C ; direct measurements of spin flavosemiquinone proportions have been carried out by EPR determinations at the same temperature. Our results show that the equilibria involving flavin are largely affected by the presence of pyruvate ; the semiquinone proportion markedly increases almost till unit near half-reduction of cytochrome b_2 ; at 10 mM pyruvate, the dismutation constant, $K_{dism} = (F_s)^2/(F_o)*(F_r)$ increases by a factor $\geq$ 10.

In respiratory chains, the redox potentials of the various carriers are one of the parameters that control the electron flow, other factors being linked either to specific interactions and/or to compartimentation. It is often assumed that the redox parameters of the carriers are essentially invariant in the cellular environment, a part from pH effects. However, this is not necessarily so and, in the flavoprotein field, there are reports of specific inhibitors provoking a change of intrinsic redox potentials with stabilization of the semiquinone form (1,2).

In this communication, we report direct evidence of a marked modification in the redox equilibria between heme and flavin which are associated in the active centers of flavocytochrome b_2 (L-lactate cytochrome c oxidoreductase EC 1.1.2.3) when the product of lactate oxidation, pyruvate, is bound to the enzyme. In this enzyme that can be classified as a dehydrogenase/electron transferase or a 2e/1e transformase according to Hemmerich and Massey (3), semiquinone is an obligatory intermediate in turnover, and has been shown to reach consistent levels (40 % of total flavin) during enzyme reduction by

Abbreviations : F_o, F_s, F_r : oxidized, semiquinone and reduced flavin respectively; H_o, H_r : oxidized and reduced heme respectively (ie cytochrome b_2).

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L-lactate or redox titrations (4). Therefore two independent one-electron couples have to be considered at the level of flavin : F_o/F_s and F_s/F_r.

The first indication that pyruvate could markedly alter redox equilibria between prosthetic groups of this enzyme was provided by T-Jump studies of the intramolecular electron transfer between flavin and heme (6).For the relaxation assigned to a one-electron transfer from F_s to H_o in the absence[1] of pyruvate,the amplitude -whatever the reduction level- was proportional to the amount of donor-acceptor couple as calculated on the basis of published redox parameters (5). However, in the presence of pyruvate (1 to 10 mM), amplitude values at different levels of reduction could be interpreted only by a change in the relative population of the various species ie of redox equilibria. In order to examine the validity of this interpretation, it was necessary to measure the equilibrium constants involved in the two redox exchanges between flavin and heme in the absence and presence of pyruvate.

An investigation was carried out in order to determine directly the equilibrium constants and hence the redox potential differences between heme and the two flavin systems, in the presence and in the absence of pyruvate, under the same conditions employed in the T-Jump studies. Therefore, EPR determinations of spin concentration as well as absorbance measurements were carried out near 20°C. The results are reported in this paper.

MATERIALS and METHODS :

- Pure L-lactate cytochrome c oxidoreductase, or flavocytochrome b_2, was prepared from Hansenula anomala yeast as described in (7) with modification in (8). Molecular activity (1000 sec^{-1},electron/cyt.b_2) and (280 nm/$\gamma peak_{red}$) absorbance ratio (0.47) were optimal. ε values are 30.9 and 21.6 $mM^{-1}cm^{-1}$ respectively for the reduced form and for the reduced-oxidized increment at 556 nm (λ max of α peak) (7).

- Flavodoxin from Desulfovibrio vulgaris was a kind gift from Dr. V.Favaudon. Concentrations of the oxidized and semiquinone forms were calculated with ε values of 10.7 and 4.1 $mM^{-1}cm^{-1}$ respectively for the oxidized form at 460 nm and the semiquinone form at 580 nm (9). Flavodoxin semiquinone was obtained by irradiation according to (9), up to total conversion, as tested by characteristic absorption.

- EPR signals were recorded with a Brucker apparatus at room temperature using a dual cavity (flavocytochrome b_2/flavodoxin semiquinone). Microwave power : 2.2 mW (proportionality between signal intensity and $\sqrt{P}$ being fulfilled up to 5 mW for the two kinds of radical), modulation amplitude : 8 Gauss, sweep range : 100 Gauss, sweep time : 2 min, time constant : 1 sec. Relative concentrations were deduced from double integrals of the signals.

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[1] As a matter of fact, in these experiments we had a stoichiometric amount of pyruvate formed by oxidation of lactate, which was added to reduce the enzyme. For the sake of simplicity we shall refer, also in this paper, to such conditions in which pyruvate is stoichiometric with enzyme, as "absence of pyruvate". This is justified by the fact that the concentration of pyruvate at which the phenomena described in this paper appear is much higher (1 to 10 mM).

RESULTS

Fig. 1 shows the variation of the flavosemiquinone proportion, F_s,(by EPR measurements) vs the percentage of reduced heme, H_r, in the absence and in the presence of 10 mM pyruvate. Defining equilibrium constants (see Table 1):

$K_1 = (H_r)*(F_o)/(H_o)*(F_s)$ for reaction 1, and

$K_2 = (H_r)*(F_s)/(H_o)*(F_r)$ for reaction 2. The theoretical curve is :

$(F_s) = 1/(1+a/K_2+K_1/a)$, with $a = (H_r)/(H_o)$ (I)

Least square fits of these curves for different pyruvate concentrations and control simulations with variable values of K_1 and K_2 have been used to obtain the best estimates with uncertainty limits. In Table 1, K_1 and K_2

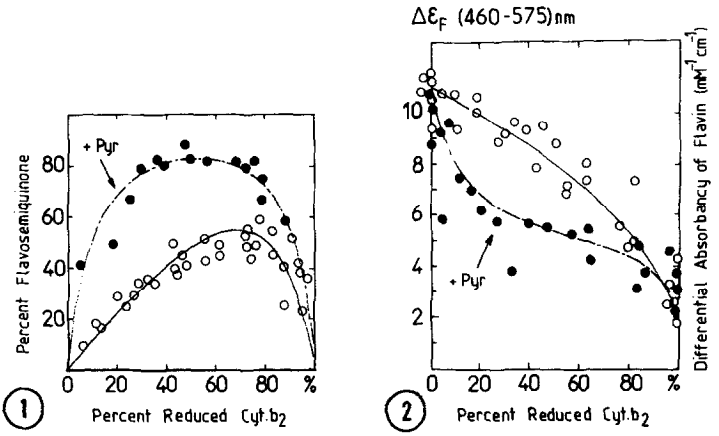

Fig. 1 : Effect of pyruvate on the proportion of radical flavosemiquinone in redox equilibrium with the cytochrome b_2 moiety (pH 7, 18°C).

Sample of oxidized flavocytochrome b_2 (100 µM) are partially reduced at various levels by addition of titrating agents (lactate or ferricyanide) (under argon flow) and transferred under argon pressure into a quartz flat EPR cell. The level of % reduced heme is measured from absorbance data at 556 nm (Cary 219) and the level of semiquinone by EPR at 18°C, by comparison with a sample of flavodoxin quantitatively converted to semiquinone by illumination in anaerobiosis; the latter proved to be perfectly stable in the course of the experiments. Circles are from three different experiments without (o) and with (•) 10 mM pyruvate. Solid lines are simulations with corresponding K_1 and K_2 values reported in Table 1.

Fig. 2 : Absorbance redox titration of the flavin relatively to heme in flavocytochrome b_2 : effect of pyruvate (pH 7, 14°C).

Samples of oxidized flavocytochrome b_2 (20 µM) are titrated in thunberg spectrophotometric vessels under argon flow cf. Fig. 1. Spectra are recorded and memorized (with the help of a microcomputer) at equilibrium (less than 1 min after addition of the titrating agent). Spectra of fully oxidized and reduced cytochrome b_2 core are also memorized. The level of H_r as measured at 556 nm is used to calculate, from cytochrome b_2 core data (10), heme absorbancy at each wavelength for this reduction level.The proper absorbancy of flavin is obtained by substraction of heme contribution from total absorbancy of the flavocytochrome . Circles are calculated values of ε_F (for 460-575 nm) (without (o) and with (•) 10 mM pyruvate) in four independent experiments. Solid lines are simulations with:
(a) proportions of F_o, F_s and F_r derived from K values listed in Table 1 and
(b) $\Delta\varepsilon$ values of 11, 6.4 and 2 $mM^{-1}cm^{-1}$ (without pyruvate) and 11, 5 and 2 $mM^{-1}cm^{-1}$ (with pyruvate) for species F_o, F_s and F_r respectively.

The titration curves of flavin relatively to heme are independent of the presence of methylene blue added as mediator.

Table 1 : Redox equilibria between heme and flavin : influence of pyruvate

	Reaction 1		Reaction 2		Reaction 3
	$F_{sq} + H_o \rightleftharpoons F_o + H_r$		$F_r + H_o \rightleftharpoons F_{sq} + H_r$		$2\,F_{sq} \rightleftharpoons F_o + F_r$
Pyruvate (mM)	K_1	ΔE_{m1}	K_2	ΔE_{m2}	$K_{dism} = K_2/K_1$
0	0.84 *(0.15)*	-5±5 mV	7.6 *(2.8)*	51±10 mV	9±5
1	0.24 *(0.04)*	-38±5 "	46 *(14)*	100±20 "	190±90
10	0.1 *(0.02)*	-60±6 "	13 *(3)*	70±10 "	130±60

K_1, K_2 are equilibrium constant of reaction 1, 2 as defined in the text. Best estimates and standard errors (in parenthesis) are derived from least square fits of equation (I). ΔE_{m1} and ΔE_{m2} are differences in midpoint potentials at pH 7 between heme (cyt b_2) and flavin $1e^-$ couples, *ie* $E_{m,heme} - E_{m,flavin}$, respectively for F_o/F_s and F_s/F_r. K_{dism} is the dismutation constant. Preliminary data, from comparison of midpoint potentials of heme and methylene blue (spectrophotometric titrations) indicate that the presence of pyruvate does not modify $E_{m,heme}$ that remains equal to-25 ± 10 mV, a value equal to that reevaluated by Capeillère-Blandin, -22 mV (personal communication) in the absence of pyruvate.

are given together with the corresponding values of ΔE_{m7}(heme-flavin), *ie* $(58 * \log_{10} K)$ in mV, and of the dismutation constants $K_{dism} = (F_s)^2/(F_o)*(F_r)$.

In the absence of pyruvate, the new results, although not identical with those obtained by Capeillère-Blandin et al (5) using the rapid-mixing and freezing technique, are the same within the uncertainty limits given by these authors. This insure that no significant changes in the concentration of the semiquinone took place during the freezing time in these experiments.

In the presence of 10 mM pyruvate, the effect on reaction 1 is very large, K_1 decreasing by ∿10 times (and thus ΔE_{m7} being ∿60 mV lower) ; however the effect on reaction 2 is much smaller and, in view of the larger errors, more uncertain, as shown in Table 1. It may be noticed that the dismutation constant changes by at least a factor of 10. In fact a preliminary investigation in which the redox potentials of each couple is compared with that of methylene blue, indicates that the E_{m7} value for the heme group of flavocytochrome b_2 (5) is not altered by pyruvate, while absolute E_{m7} values of the two flavin one e^-couples are affected by pyruvate. These results taken together show that the semiquinone is thermodynamically "stabilized" reaching higher levels upon pyruvate binding.

In parallel with these redox titrations involving EPR measurement of the radical, spectral titrations were carried out with the purpose of calcula-

ting, for each reduction level of flavocytochrome b_2, the spectral contribution of the flavin. Fig.2 presents the decrease in the differential absorption of the flavin $\Delta\varepsilon = \varepsilon_{460} - \varepsilon_{575}$ nm as a function of $\% H_r$, in the absence and in the presence of 10 mM pyruvate. Simulations taking into account typical absorbances for an intermediate semiquinone (11), as well as K_1 and K_2 values given in Table 1, perfectly fit experimental data. The experiments in the presence of pyruvate show a large plateau between 30 and 80% heme reduction suggesting the accumulation of a stable one-electron intermediate ; comparison with Fig.1 indicates that, actually in this reduction range, flavosemiquinone represents over 80 % of total flavin when pyruvate is present but less than 40 % in its absence. Under such conditions, the intermediate flavin spectra are very similar to the flavosemiquinone spectrum itself ; the latter can be better estimated by correcting for the contributions of the other forms present (ie F_o or F_r) on the basis of results presented in Fig.1. Such a spectrum (Fig.3) presents a peak at 486 nm (mean value : $\Delta\varepsilon_{486-575} = 5 \pm 1.0$ $cm^{-1}mM^{-1}$) and no significant absorbance above 560nm, features characteristic of a red ($\Delta\varepsilon \sim 5$ $mM^{-1}cm^{-1}$) and not a blue ($\Delta\varepsilon \leq 1$ $mM^{-1}cm^{-1}$) semiquinone (11). The semiquinone was previously assumed to be of the red type because of the linewidth of 15 gauss found in earlier

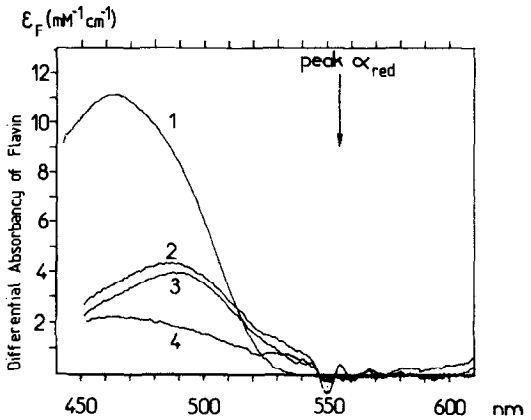

Fig. 3 : Calculated spectra of different forms of the flavin in the presence of 10 mM pyruvate.

Flavin spectra are calculated from memorized spectra cf. Fig. 2 for fully oxidized, dithionite reduced and partially reduced flavocytochrome b_2 samples in one typical experiment.

Spectra 1 to 4 are computed tracings of flavin extinction coefficients, (in terms of $\varepsilon_\lambda - \varepsilon_{575}$) corresponding to the following flavocytochrome b_2 samples: 1) F_o in a fully oxidized sample ; 2) F_r in a dithionite reduced sample ; 3) F_m (ie apparent flavin absorbancy) in a sample with 84% reduced heme, about 85% F_s and 15% F_r ; 4) F_s, ie calculated absorbancy of the semiquinone in a sample with 34% reduced heme, assuming 90% F_s and 10% F_o.

It should be noted that the too large relative absorbance of reduced heme at 556nm ($\varepsilon = 30.9 mM^{-1}cm^{-1}$) precludes precise correction in the range 545-565 nm ; outside this range, the limits of uncertainty stand lower than ± 1 $mM^{-1}cm^{-1}$.

determinations at -150°C after rapid-freezing, on the basis of the correlation established by Palmer et al (12) ; a linewidth of 15 gauss is also found in the present study at 18°C in the absence or the presence of pyruvate.

As can be seen in Table 1, the effect of pyruvate on the dismutation constant is already maximal at 1 mM while K_i value, in a range where steady-state inhibition behaves as competitive, is near 5 mM (Tegoni, unpublished data). This suggests that affinity of pyruvate for the semiquinone form of the enzyme is significantly higher than for the oxidized form. The detailed implication of the effect of pyruvate binding to the semiquinone form of the enzyme and its consequences in terms of modulation of the dynamics of catalysis and that of the individual rate constants involved in the various electron transfers will be discussed elsewhere. However it can be noted that the observed complex inhibition pattern ((13) and Tegoni, unpublished data) might be due to the fact that pyruvate binds not only competitively with the substrate to the oxidized enzyme, but still more strongly to a semiquinone intermediate that is consequently highly stabilized as a sort of "dead-end" complex. The reported inhibition by excess lactate might also corresponds to a "dead-end" complex formed with the flavosemiquinone protein, a complex in which lactate cannot be dehydrogenated.

Interaction of pyruvate with the enzyme is certainly not of the "allosteric"-type but should correspond, according to all available data on flavoproteins (14) to a direct contact between the α-carbon of the protein-bound pyruvate located above the plane of the isoalloxazine ring, and the flavin position N(5). Indeed, pyruvate as well as oxalate are able to bind covalently to the flavin of L-lactate cytochrome c reductase (ie flavocytochrome b_2) ((15) and Blazy personal communication) as they do to lactate oxidase of Mycobacterium smegmatis (16).

In conclusion, on the basis of our present understanding, accumulation of pyruvate will stabilize the enzyme in an unreactive form -a semiquinone-pyruvate complex ; thereby, it not only affects to a considerable extent the kinetics of lactate dehydrogenation even when lactate remains at high relative concentration, but also the kinetics of the internal electron transfers (6) taking place between the flavodehydrogenase and cytochrome b_2 domains of this complex enzyme (16). Such an effect may be more general than anticipated and is possibly, at least at the level of flavoreductases, one way of regulation for the respiratory system as suggested by the increase in semiquinone thermodynamic stability upon oxaloacetate binding to succinate dehydrogenase already reported by Gutman et al (1).

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