A PULSE RADIOLYSIS STUDY OF FLAVOCYTOCHROME b_2 : DIFFERENCES IN REACTIVITY WITH CARBOXYLATE RADICALS BETWEEN FLAVIN AND HEME b_2.

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Introduction

Reduction processes of flavocytochrome b_2 by a 2-electron donor, L-lactate, have already been studied at equilibrium and in the course of rapid kinetic studies (1,2). The electron transfer sequence starts by the 2-electron reduction of flavin by bound lactate within the flavodehydrogenase moiety followed by a reversible intramolecular 1-electron transfer between flavin hydroquinone and heme b_2, the latter belonging to the cytochrome b_2 domain with the concomitant formation of flavin semiquinone. Further reversible intramolecular electron transfer between semiquinone and cytochrome b_2 has been shown in T-jump studies on partially reduced enzyme (3). To bring additional information to the understanding of electron transfer processes inside such a multi-centered redox proteins we studied transient states of Hansenula anomala flavocytochrome b_2 obtained by reduction with a 1-electron donor of low potential, at a 1000 fold higher time resolution than with conventional rapid mixing techniques, using the radical $CO_2^{\cdot-}$ generated by radiolysis.

Results

1) Transient absorbance difference spectra. We carried out a detailed spectral investigation (280-600 nm) of the reduction products of oxidized

Flavins and Flavoproteins

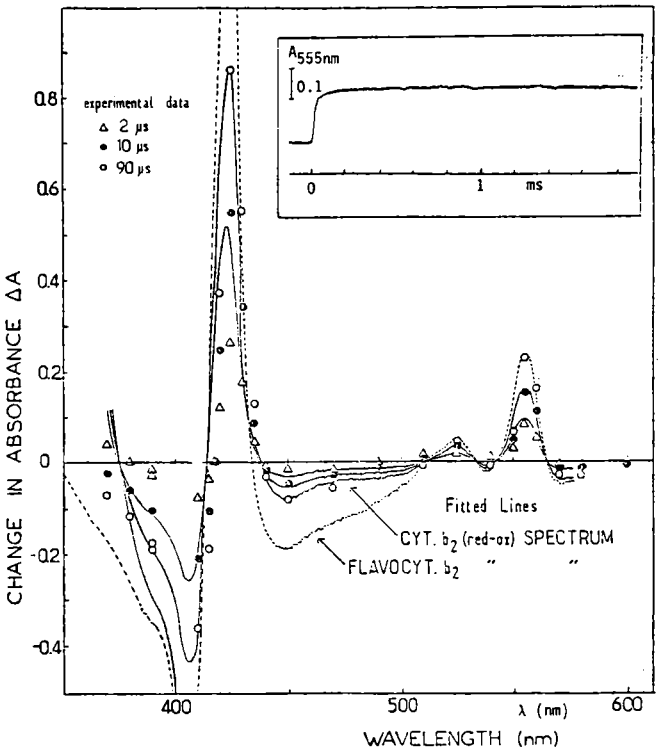

Fig.1. Total absorbance changes measured 2, 10 and 90 μs after the pulse in the course of the reaction between flavocytochrome b_2 and $CO_2^{\cdot-}$ in 2 mM phosphate buffer pH 7.0 and 0.16 M formate solution saturated with N_2O at 20°C. Solid lines passing through the points (600-400 nm) represent the (red-ox) spectra of cytochrome b_2 obtained by normalisation in the α band for the different reaction times. The dotted line corresponds to the (red-ox) spectrum of flavocytochrome b_2 normalized at 90 μs. Insert : absorbance recording of the heme b_2 reduction followed up to 2 ms at 555nm.

flavocytochrome b_2 (20 μM) by $CO_2^{\cdot-}$ produced at a (450±50)μM concentration in less than 1 μsec (4). The transient absorbance difference spectra (Fig. 1) above 400 nm agreed quantitatively with the characteristic spectral features of heme b_2 as detected in the difference spectra of cytochrome b_2 core, isolated from flavocytochrome b_2. Below 400 nm the additional absorbance of the $CO_2^{\cdot-}$ prevents the fit. Over the whole spectrum no further changes were observed up to 2 ms : in particular at 0.3 ms when $CO_2^{\cdot-}$ has been consumed,heme b_2 was 47 % reduced and remained apparently at this level (Fig.1 insert). Nevertheless the formation of 20 % semiquinone could

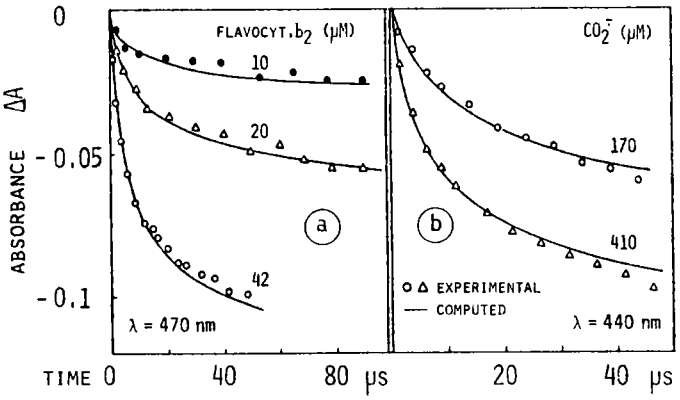

Fig. 2 : Dependence of the reaction time-course on the initial concentrations of oxidized flavocytochrome b_2 (a) and $CO_2^{\cdot-}$ (b). The solid lines have been computed using scheme I for the following best-fit parameters : $2k_1$ = 1.1 x 10^9 $M^{-1}s^{-1}$ and from top to bottom in (a) k_2 = 2.2 x 10^8 $M^{-1}s^{-1}$ with $\Delta\varepsilon$ = -5, -5.5 and -5.3 $mM^{-1}cm^{-1}$; in (b) k_2 = 2.2 x 10^8 $M^{-1}s^{-1}$ with $\Delta\varepsilon$ = -4.2 $mM^{-1}cm^{-1}$ and k_2 = 2.4 x $10^8 M^{-1}s^{-1}$ with $\Delta\varepsilon$ = -3.6 $mM^{-1}cm^{-1}$.

escape absorbance detection. Therefore it appears that the bound heme b_2 is preferentially reduced by $CO_2^{\cdot-}$.

2) Kinetic analysis of the reaction. The proportion of reduced flavocytochrome b_2 determined a constant time after the pulse (t = 50 μs) were found, to be independent of the observation wavelength (fig. 1) but to vary with initial concentrations of $CO_2^{\cdot-}$ (170-500 μM) and of flavocytochrome b_2 (10-46 μM) (Fig. 2). These reaction time-courses were fitted using scheme 1 i.e taking into account two simultaneous second-order reactions: the self-recombination of $CO_2^{\cdot-}$ ($2k_1$) and the reduction of heme b_2 bound to flavocytochrome b_2 by carboxylate radical (k_2).

The fitting of the computed curves to the experimental data was obtained using absorbance difference coefficients within the experimental uncertainty limits for cytochrome b_2 : $\Delta\varepsilon_{440}$ = -3.2(∓0.7) $M^{-1}cm^{-1}$; $\Delta\varepsilon_{470}$ = -4.6(±0.5) $M^{-1}cm^{-1}$ and reduction second-order rate constant k_2 = (2.1 ± 0.2)x10^8 $M^{-1}s^{-1}$. The close fit between experimental points and computed curves (Fig.2) strengthens the validity of such a kinetic model involving two second-order processes.

Discussion

In free enzyme molecules the protein-bound heme b_2 is directly reduced by $CO_2^{\cdot-}$ while the protein-bound flavin does not seem to be significantly reduced. These differences in reactivity with $CO_2^{\cdot-}$ between flavin and heme b_2 might indicate surface structural differences affecting the relative exposure of each prosthetic group to the solvent. Whereas the heme b_2 crevice would be accessible the flavin isoalloxazine ring would be buried, prevented from solvent contact by a compact folding of the polypeptide chain.
The mechanism of reduction of flavocytochrome b_2 by $CO_2^{\cdot-}$ which proceeds first by the heme b_2 attack, is different from that proposed for the catalytic electron transfer reaction where the reduction of flavin by bound lactate is the first step followed by an electron distribution among both prosthetic groups (1,2). The absence of detectable electron transfer between reduced heme b_2 and flavin, here observed on a short time scale $\lesssim 10$ ms, points out that under our experimental conditions such a transfer is slowed down and no longer equal to 200 s^{-1} (3). The modification of the reactivity of flavin might be considered as an evidence of the existence of a possible mobility of the two structural domains of the molecule : in the free enzyme molecules random molecular motions could prevent the two electroactive groups from reaching the right position and orientation allowing electron tunneling. On the contrary when the substrate (or product) is bound the two domains may be subjected to constraints which would reduce the amplitude of such motions and thus might facilitate the electron transfer. However the use of high ionic strength conditions can be taken into consideration.

References

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