THE CIRCULAR DICHROISM AND OPTICAL ABSORBANCY OF THE HISTIDYL FLAVIN DURING ACTIVE–NON-ACTIVE TRANSITION OF SOLUBLE SUCCINATE DEHYDROGENASE

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1. Introduction

Oxaloacetate is a negative effector for succinate dehydrogenase having a major role in the process of activation, i.e., the reversible transition of the enzyme between a catalytically inactive and an active form. The stable form of the non-active enzyme is a complex with oxaloacetate, in a 1:1 ratio to flavin, and the unchanged modulator is dislodged only by denaturation of the protein or by any type of activation [1–4]. In [4] it was proposed that the substrate binding site and the regulatory site are not the same and oxaloacetate does not form a thiohemiacetal linkage with an SH group essential for enzymic activity as suggested [5,6] but it induces a change in conformation of the enzyme.

The nature of the non-active form of the enzyme was deduced from reductive activation titrations [7]. Quantitative analysis of such titrations [8] led to the conclusion that the redox potential of the flavin in the active enzyme equals 6 ± 20 mV (compatible to act as oxidant with respect to succinate). On the other hand the redox potential of the flavin in non-active enzyme is −186 ± 20 mV. Such a low redox potential is compatible with that of free flavin (−167 mV for the isolated histidyl flavin of succinate dehydrogenase [9]) and accounts for the fact that non-active enzyme can not be reduced by succinate unless the enzyme undergoes activation.

The shift in the redox potential of the flavin was attributed to a conformation change of the flavin moiety [8], such as bending of the planar form of oxidized [10] or semiquinone [11] flavin to the non-planar structure of reduced flavin [10]. Molecular orbital calculations indicated that the energy associated with such transformation would suffice to shift the redox potential by such an amount. A different mechanism accounting for such a shift, and which also accounts for the fact that activation by reduction is a one electron reduction [8], is by interaction of polar groups with N_1 or N_5 of the isoalloxazine ring [12].

Whatever is the mechanism shifting the potential of the flavin, both predict a major change in its CD spectrum. The isoalloxazine ring is devoid of optical activity unless asymmetry is introduced by interaction with the ribityl hydroxyls or with protein side chains [13].

As documented here, a soluble, purified reconstitutively active succinate dehydrogenase demonstrates major changes in its flavin (and FeS) CD spectra when transformed between its active and non-active states.

2. Materials and methods

Succinate dehydrogenase was purified as in [14]. In our studies we used the enzyme either after gel filtration (1 mol histidyl FAD/260 000 g protein) or after DEAE–Sephadex chromatography (1 mol histidyl FAD/97 000 g protein); the former was preferred in somewhat longer experiments because of its enhanced stability. Still the results with both types

of enzyme were the same. The reconstitutive activities of the preparations were 100 and 40%, respectively.

The ammonium sulphate precipitate of the enzyme at the purification stages mentioned was gel filtered and aliquots of the protein in 50 mM Tris–acetate buffer (pH 7.5) containing 5 mM succinate were frozen in liquid nitrogen and stored. Each experiment was run on a newly thawed sample.

Activity of the enzyme was measured spectrophotometrically with 2 mM phenazine methosulphate and 0.08 mM 2,4-dichlorophenol indophenol.

The amount of oxidized enzyme at various succinate to fumarate ratios was calculated from the corresponding redox potential and the potential of the flavin in the active enzyme [8].

Absorbance spectra were recorded at 12°C in a Cary 118 C spectrophotometer and circular dichroism spectra at 20°C in a Jobin-Yvon Dichrographe III (Roussel-Jouan), the cell compartment being thoroughly flushed with nitrogen.

Proteins were determined with a biuret method [15] and histidyl-FAD as in [16].

Oxaloacetate was from Fluka; fresh solutions were prepared daily in 50 mM Tris–acetate buffer (pH 7.5).

3. Results and discussion

The purpose of this study was to measure the spectral properties of active and non-active (oxaloacetate complex) enzyme. As reduction of the enzyme is expected to shift the configuration of the flavin [9] thus affecting the CD of the flavin and of the iron–sulfur centers [17,18], it was of great importance to differentiate between changes associated with activation and those reflecting redox reactions which may result from the change in redox potential of the flavin. Because of this we preferred to stabilize the active enzyme by complexing it with fumarate, to ensure its oxidized state. Addition of oxaloacetate to such preparations will deactivate the enzyme with the flavin already oxidized.

The kinetic analysis of the association of malonate and of oxaloacetate with succinate dehydrogenase in submitochondrial particles has allowed differentiation between a substrate binding site and regulatory site: the ratio of dissociation constants of the ligands from the regulatory site is 80 [4]. By equilibrating the soluble enzyme with known concentrations of malonate and of oxaloacetate and determining the fraction of active enzyme [4], we measured for soluble enzyme the same ratio. This information allowed us to modulate the activation state of the enzyme by appropriate concentrations of the ligands.

The effect of deactivation on the absorbance spectrum of succinate dehydrogenase is given in fig.1. Addition of fumarate to $E' = +56$ mV led to 99.3% oxidation of the succinate-reduced enzyme. Addition of oxaloacetate (1.2 mM, enough to deactivate > 99%

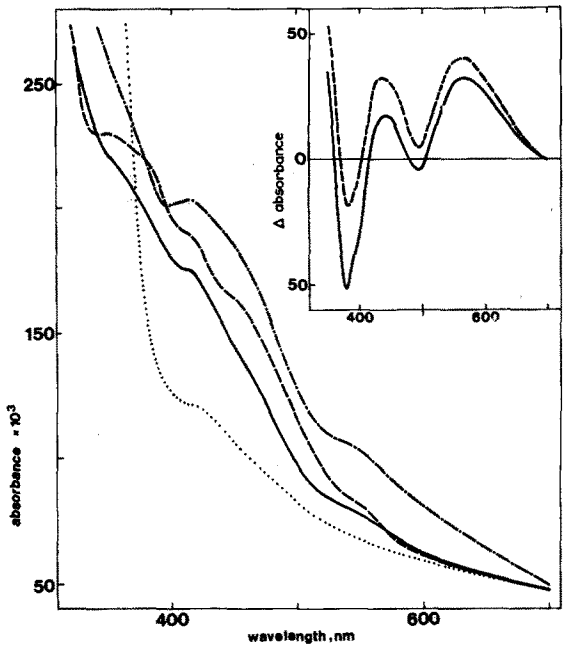

Fig.1. Effect of oxaloacetate and of fumarate on the absorbance of succinate dehydrogenase. Succinate dehydrogenase (0.6 ml) 3.22 mg .ml^{-1} (3.85 nmol his-FAD .mg^{-1}) in 50 mM Tris–acetate buffer (pH 7.5) and 5 mM succinate were measured as such (solid line) and after addition of 50 μl 0.5 M Na-fumarate in water pH 7.5 (dashes) or 7 μl 0.1 M oxaloacetate (dashes and dots); this spectrum was recorded 20 min after adding the effector. The same recording was obtained after adding 7 μl 0.1 M oxaloacetate to the fumarate-treated enzyme. The spectrum of the enzyme in succinate after addition of solid dithionite is also shown (dots). Inset: difference spectra of succinate dehydrogenase at the DEAE–Sephadex purification stage (3.12 mg .ml^{-1}, 10.28 nmol his-FAD .mg^{-1}); solid line: enzyme in succinate versus same, oxaloacetate added as above. Dashes: enzyme in succinate and fumarate versus same, oxaloacetate added as above. Both spectra were recorded 3 min after adding oxaloacetate.

of the enzyme) produced typical spectral changes (see insert) which clearly are not accounted for by oxidation of the residual 0.7% reduced enzyme. The final spectrum is the same whether oxaloacetate was added to oxidized or reduced enzyme: but as oxidation by itself caused spectral changes, the same final spectra measured in presence of oxaloacetate ± fumarate implies that during deactivation the enzyme reached its oxidized state. The same results were obtained with the two stages of enzyme purification.

As predicted by the values of dissociation constants from the regulatory site [4] excess succinate, or fumarate, added to the dehydrogenase either immediately after oxaloacetate or when the spectral modifications were completed, did not change the effect of oxaloacetate. Nevertheless dithionite restored the spectrum of the fully reduced enzyme.

As can be evaluated from the spectra the oxidation was of iron–sulfur centers as well as of the flavin moiety; similar oxidation of iron–sulfur centers has been observed [19]. The effect of oxaloacetate on the spectrum of succinate dehydrogenase has been reported before. The difference between our spectra and those in [20,21] is attributed to the fact that their starting preparations were in an oxidized state and had been prepared either completely or partly in the absence of succinate.

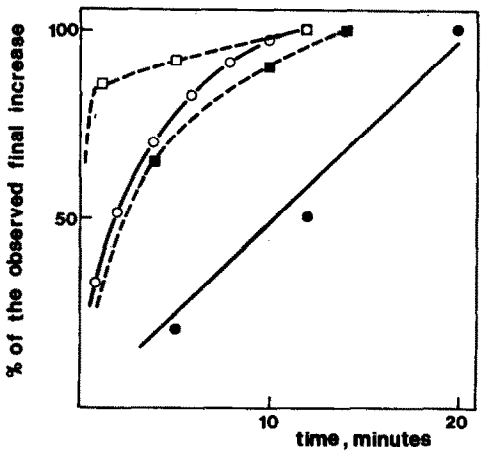

Fig.2. Time course of oxaloacetate-induced changes in absorbance (open symbols) and in circular dichroism (full symbols) at 470 nm. Oxaloacetate was added as in fig.1 to the enzyme in succinate (solid line) and to the fumarate-containing sample (dashes). The enzyme had 3.85 nmol his-FAD $\cdot mg^{-1}$.

The dynamics of the absorbance changes following addition of oxaloacetate (1.66 mM) to (5 mM) succinate-reduced enzyme are given in fig.2. Once oxaloacetate is added there is a continuous, pseudo first order, increase of absorbance of the flavin (470 nm) band ($k = 0.108\ min^{-1}$). If the enzyme was equilibrated with fumarate (50 mM) plus succinate (5 mM) the changes following oxaloacetate additions were much faster, ~80% of the reactions being completed before the first spectrum was recorded (< 30 s). The residual response had the same rate constant. These results indicate that the absorbance changes represent two reactions, one accelerated by presence of oxidant (fumarate) and a slower one which proceeds at the same rate in presence or absence of fumarate.

The CD spectra of succinate dehydrogenase are given in fig.3. This is a complex spectrum consisting of bands at wavelengths characteristic for absorption of flavin and iron–sulfur centers. Figure 3A depicts the modification in the CD spectrum following addition of oxaloacetate to active enzyme reduced by succinate. In this case we observe a response combined of changes caused by oxidation and deactivation. Due to the complexity of the CD spectrum of flavins (6 independent CD bands [13]) a quantitative analysis is still impossible. Still, looking at the changes at 470 nm, typical for the flavin (fig.2 solid symbols), we can tell that they are affected by the presence of oxidant. If succinate only is present, a slow linear increase is observed. The presence of fumarate (50 mM) alters the time curve of the response and accelerates it. These results indicate that by such a technique we observe both oxidation and deactivation.

Once the contribution of oxidation and deactivation was recognized the experimental procedure was modified so as to separate the two variables. As seen in fig.3B, the effect of addition of low (0.5 mM) and high (50 mM) fumarate concentrations to active succinate-reduced enzyme were first recorded in order to obtain the spectrum of 99.4% oxidized ($E' = +59$ mV) active enzyme. After that, oxaloacetate (1.66 mM, enough to cause > 99% deactivation) was added and the spectrum re-recorded.

Oxidation is accompanied by a dramatic increase in ellipticity corresponding to $\Theta = +10\,000$ at 480 nm (fig.3B) which appears to involve the flavin chromophore. Other conspicuous positive changes are at wavelengths of oxidized 2 iron–2 sulfur centers, like

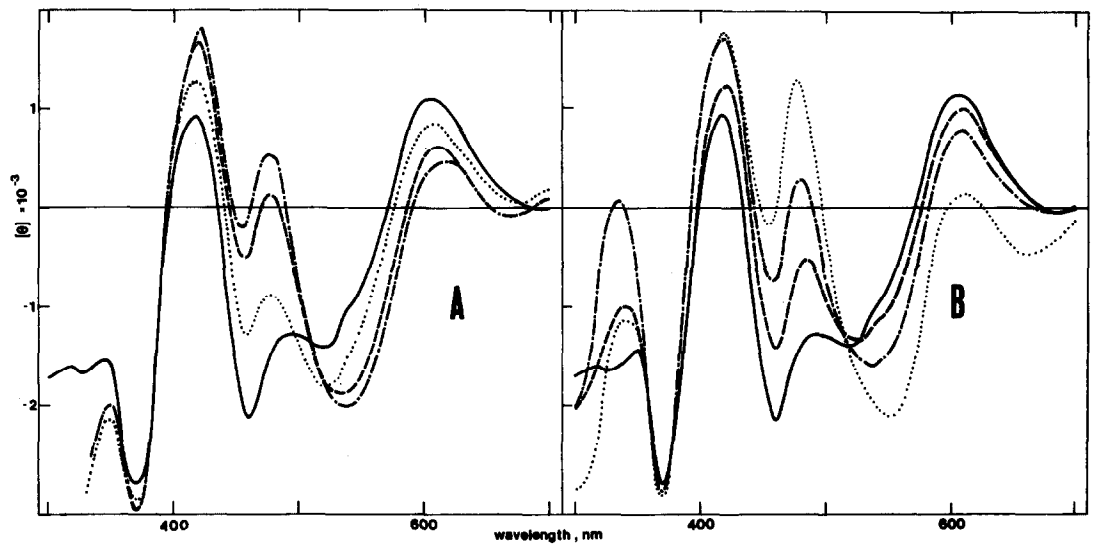

Fig.3. Circular dichroism spectra of succinate dehydrogenase at the DEAE–Sephadex purification stage. (A) Time course of oxaloacetate-induced modifications. Solid line: enzyme in succinate, as inset fig.1; 10 μl 0.1 M oxaloacetate were then added and the spectra run from 700 nm on, immediately (dots) and after 10 min (dashes) and 20 min (dashes and dots). Scanning rate was 0.5 nm .s^{-1}. (B) Modifications induced by fumarate and by oxaloacetate. Solid line, as in (A).Sequential additions were: dashes: 6 μl 50 mM fumarate; dashes and dots; 60 μl 0.5 M fumarate; dots: 10 μl 0.1 M oxaloacetate. Spectra were recorded immediately after each addition. -

420 nm whereas at those of reduced 2 iron–2 sulfur centers, e.g., 320–330 nm and 473 nm [18] the ellipticity decreases.

Deactivation of oxidized enzyme further increased the ellipticity at 480 nm, Θ = +4200 and at 550 nm, and decreased it at 330, 473 and 600 nm, these latter changes all being due to reduced tetrahedral iron chromophores [18]. Though the changes can not be subjected to quantitative analysis, they certainly indicate a major modification in the immediate vicinity of the flavin and of the iron–sulfur centers. Considering the fact that while modulating the enzyme's activity, oxaloacetate interacts with a site different from that where the substrate is oxidized [4,8], the changes in environment of flavin should be attributed to interaction of protein with the flavin moiety. The fact that addition of oxaloacetate induces oxidation of the flavin (fig.1) and shifts the redox potential of the flavin [8] suggests that these protein–flavin interactions are responsible for the alteration of the prosthetic group's properties. Our finding correlates these two observations in a model where the protein–isoalloxazine interactions modify the redox potential of the flavin as an efficient mechanism for turning off the catalytic activity. Whether the shift in redox potential is through bending of the flavin structure along the N_5–N_{10} axis [8] or through interactions of polar groups with the N_1 or N_5 sites [12] is a subject for further studies.

Acknowledgements

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