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MODULATION OF THE FLAVIN REDOX POTENTIAL AS MODE OF REGULATION OF SUCCINATE DEHYDROGENASE ACTIVITY

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Summary

The redox properties of flavin in active and non-active (oxaloacetate reacted) soluble succinate dehydrogenase were studied.

Quantitative analysis of reductive activation titrations of redox titrations of active and non-active enzyme reveal that the redox potential of the histidyl-flavin in the active enzyme (—3 ± 15 mV) is high enough to allow reduction by succinate, whereas in the non active enzyme it is —196 ± 19 mV, far to low to be reduced by substrate.

The flavin radical in the active enzyme attains 60% of total flavin at a poised redox potential of about +60 mV, upon addition of oxaloacetate the magnitude of the signal is diminished and the potential where it reaches maximal concentration is shifted by about —200 mV.

A mechanism is proposed which ascribes the fundamental difference between active and non-active enzyme to the inability of the latter to be reduced by substrate.

Introduction

Mitochondrial succinate dehydrogenase is subjected to rigorous regulation, adjusting its activity with respect to the metabolic state of the mitochondria [1,2]. This regulation is achieved by a single negative modulator, oxaloacetate [3—5], and a variety of positive modulators: substrates [5,6], anions [3,4], reduced quinone [7], ATP [1], and reduction [8,9] (for review, see Ref. 10).

Abbreviation: OAA, oxaloacetic acid.

In all cases, the non-active enzyme is identified with the enzyme-oxaloacetate complex. The role of the activators is to form stable complexes with the active form of the enzyme, a complex which prevents the enzyme from reacting with oxaloacetate.

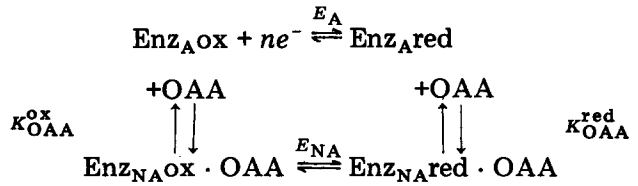
In the past years kinetic and equilibrium constants for the reactions with some activators have been measured [4.5.8], still at molecular level the mechanism rendering the non active enzyme to be as such is not clear. The problem why non-active enzyme does not catalyze the redox reaction, though reductant and oxidant are present, is the subject of the present paper.

In an effort to unify the various specific mechanisms in a single mode the fundamental difference between active and non-active enzyme is ascribed to the inability of the latter to be reduced by substrate.

The reduction of the histidyl-flavin of succinate dehydrogenase by succinate is known for many years [6]. Considering the fact that the redox potential of the substrate (E'_0 = +30 mV, 25°C [11]), we must conclude that the flavin in the enzyme assumes a redox potential much higher than that of free flavins (—200 mV [11]) or that of histidyl-flavin (—170 mV [9]). Consequently there should be a mechanism which raises the redox potential of the covalently bound histidyl-flavin to the redox potential of the substrate.

Non-active enzyme is not reduced by succinate, neither by malate (E'_0 = —160 mV [11]). Years ago Van Voorst et al. [12] reported that malate caused a transient reduction of the enzyme. This observation is now explained by the fact that malate is ozidized by the enzyme to oxaloacetate with concomitant deactivation [13,14]. Once the enzyme is in the non-active form, malate can not reduce it anymore. Combination of these observations suggests that activation corresponds to a shift of the redox potential of the flavin from a potential lower than that of malate to a value approximating the potential of the substrate, a difference of about 200 mV.

Nonactive enzyme is readily reduced by strong reductants like dithionite. This reaction is accompanied by activation [8,9,15,16], but the extent of activation is a function of two variables: the applied redox potential and oxaloacetate concentration [8,17]. At high redox potential very low concentrations of oxaloacetate suffice for deactivation, but upon lowering of the applied potential higher concentrations of oxaloacetate are needed for deactivating the enzyme. The coupling between the activation and reduction of the enzyme is given by Scheme I [10,17].



Scheme I.

where E_A and E_{NA} are redox potentials of the active and non-active forms of the enzyme while K^{ox}_{OAA} and K^{red}_{OAA} are the respective dissociation constants of the reduced and oxidized complexes.

Ackrell et al. [9] estimated $K^{red}_{OAA}/K^{ox}_{OAA} = 10$ and using the equation

$$E_{app} = E'_{0A} - \frac{60}{n} \log \frac{1 + (OAA/K^{ox}_{OAA})}{1 + (OAA/K^{red}_{OAA})} \quad (1)$$

concluded that $E'_0A = -90$ mV. This potential is far too low to account for the reduction of the flavin by succinate. As shown in the present paper quantitative analysis of reductive activation titrations or redox titrations of active and non-active enzyme reveals that the redox potential of the histidyl-flavin in the active enzyme (6 ± 20 mV) is high enough to permit reduction by succinate. On the other hand the redox potential of the flavin of non-active enzyme is −195 ± 20 mV, far too low to be reduced by substrate.

Materials and Methods

Succinate dehydrogenase was purified from beef heart as previously reported [18] either up to the DEAE-Sephadex eluate step for spectrophotometric measurements, or to the gel eluate step for EPR studies. The flavin content and reconstitutive activity of the two preparations were: one mol histidyl-FAD per 97 700 g protein and 40% reconstitutive activity and one mol per 260 000 g and 100% respectively.

The ammonium sulphate precipitate at the mentioned steps of purification was gel filtered and aliquots of the protein, in 50 mM Tris acetate buffer (pH 7.5) containing 5 mM succinate, were stored in liquid nitrogen. Each experiment was run on a newly thawed sample. As succinate was present at all purifucation steps the enzyme was fully activated. Activity of the enzyme was measured spectrophotometrically with 2 mM phenazine methosulphate and 0.08 mM 2,4-dichlorophenol indophenol.

Redox titration for EPR were performed in an argon atmosphere. The enzyme dissolved in 5 mM succinate was titrated by addition of known amounts of fumarate or freshly prepared, oxygen-free, solutions of dithionite. Redox potentials were either calculated from the ratio of succinate to fumarate or were measured with a combined platinum silver chloride electrode. The transfer of the samples from the titration vessel to the EPR tubes was carried out also under argon.

Absorbance spectra were recorded at 13°C in a Cary 118C spectrophotometer, the cell compartment being thoroughly flushed with nitrogen.

Proteins were determined with a biuret method [19] and histidyl-FAD as previously described [20].

Oxaloacetate was purchased from Fluka; fresh solutions were prepared daily in 50 mM Tris acetate buffer, pH 7.5.

Results

Determination of redox potential of flavin by reductive activation

Based on many titrations [8,10,17] we can approximate $K^{ox}_{OAA} = 0.2$ μM and $K^{red}_{OAA} = 400$ μM. Assuming these values, we used Eqn. 1 given by Ackrell et al.

[9] and the corresponding expression for the non-active enzyme [10]

$$E_{app} = E'_{0NA} - \frac{60}{n} \log \frac{1 + (K^{ox}_{OAA}/OAA)}{1 + (K^{red}_{OAA}/OAA)} \quad (2)$$

to analyze the experiments of Gutman and Silman [8,17]. The results are given in Table I. It is evident that the estimated potentials of active and non-active enzyme are constant, though they were calculated from experiments carried over a very wide range of ligand concentration (2000 folds) and of redox potentials (+55 to −200 mV).

As seen from Table I, reductive activation seems to be a one electron reaction. Recalculation of figures assuming $n = 2$ (as done by Ackrell et al. [9]) yields inconsistent results (values in parenthesis).

Redox titration of the flavin of active succinate dehydrogenase

As implied by our calculation the redox state of the flavin in active enzyme should be titrated by the fumarate succinate couple. The enzyme was activated by succinate and absorbance at 450 nm was measured under N_2 atmosphere after addition of graded amounts of fumarate. As soluble reconstitutively active enzyme is unstable in the absence of succinate, we could not reverse the direction of the titration. The procedure of replacing the succinate (in which the enzyme is stored) by fumarate is time consuming leading to substantial loss of

TABLE I

ESTIMATION OF REDOX POTENTIALS OF THE FLAVIN IN ACTIVE AND NON-ACTIVE SUCCINATE DEHYDROGENASE

The values were calculated from the data given in Fig. 7 of Ref. 10 using Eqns. 1 and 2. The values tabulated were calculated for $n = 1$. Examples of potentials calculated with $n = 2$ are given in parenthesis. The rows marked with + are the results of Ackrell et al. [9].

E_{app} (mV)	Oxaloacetate (μM)	E'_{0A} (mV)	E'_{0NA} (mV)
−195	400	−15 (−105)	−213 (−204)
−180	250	−7	−203
−172	100	−16	−214
−168	100	−12	−210
−160+	67	−13	−210
−160+	202	+9	−188
−150	20	−32	−229
−100	10	1 (−49)	−196 (−148)
−60	2	2	−195
−50	1.9	11	−187
−40	1.7	18	−179
−40	2.2	24	−173
−28	0.8	14	−184
−20	1.0	27 (−23)	−171 (−99)
0	1.0	47	−151
0	0.7	39	−159
20	0.35	−6 (+7)	−191 (−105)
28	0.25	7	−205
33	0.37	−3	−200
55	0.2	37	−235
		6 ± 20 mV	−196 ± 19 mV

activity. For this reason we had to limit our measurements only to the oxidative direction of the redox titration. Still, in order to ensure that after each addition of fumarate equilibrium was established, we monitored the absorbance changes (450 nm) following each addition of fumarate. Only after the reading became stable, the absorbance spectra were recorded (700—300 nm). Enzymic assay at the end of the titration indicated that the enzyme retained more than 90% of its activity.

At 450 nm the FeS centers are also absorbing and their contribution varies with the absorbance of the FeS centers by calculating the difference spectrum of oxidized vs. *p*-chloromercuribenzoate-treated enzyme (i.e. absorbance of oxidized FeS centers) minus the spectrum of dithionite treated enzyme (i.e. absorbance of reduced FeS centers). The amount reduced at the different potentials was calculated using the redox potential given by Ohnishi [21]. The results were subtracted from the measured values. The corrected and uncorrected data are both presented in Fig. 1. If we consider the absorbance of 450 nm as pure contribution of the flavin, then the results are compatible with a redox titration of a two electron acceptor with mid potential of −15 mV. The correction for the contribution of the FeS centers to the absorbance changes at 450 nm, shifts the mid potential to −3 mV but the uncertainty of the redox

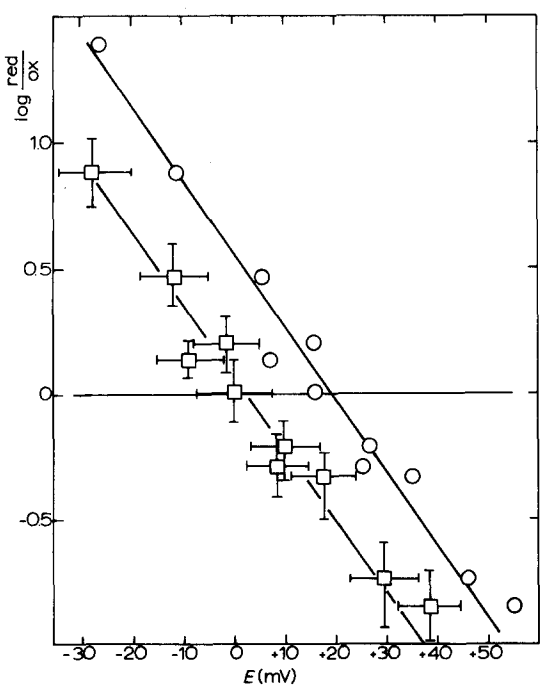


Fig. 1. Redox titration of the flavin in soluble succinate dehydrogenase. The enzyme, 1.65 mg · ml^{-1}, 10.27 nmol His-FAD · mg^{-1} protein, 40% reconstitutive activity, in 50 mM Tris acetate buffer (pH 7.5) and 5 mM succinate, was titrated under nitrogen by adding known amounts of fumarate at 13°C. The absorbance at 450 nm was measured, until reading reached a constant value and the absorbance spectrum was measured (700—300 nm). In order to correct for loss of absorbance on aging the reference cell contained the same preparation and buffer was added instead of fumarate. Data were corrected for dilution. The results are presented as Nernst plot for absorbance changes at 450 nm either uncorrected (○) or corrected for the contribution of the Fe_2S_2 center S_1 to the absorbance changes at this wavelength (□).

potential of the FeS centers introduces inaccuracy to the measured value so that our estimate is −3 ± 15 mV. It should be emphasized that either value is some 200 mV more positive than the redox potential of free flavins [11]. These high redox potentials are within the range predicted by the proposed model for reductive activation [10], and in accord with Table I.

EPR redox titration of the flavin in active and non active enzyme.

The implication of the redox-control model for regulation of the enzymic activity by oxaloacetate is that the two states of the enzyme will differ by 200 mV in their redox potential. Furthermore, as activation coincides with the reduction of the flavin [9] it implies that the component shifting its redox potential by that magnitude should also be the flavin [10]. As seen in Fig. 2 the presence or absence of oxaloacetate had a major effect on the redox behaviour of the flavin radical. In active enzyme the free radical is observed at fairly high redox potentials reaching a maximum at applied potential of approx. 60 mV. The bell shape response of the free radical to the redox potential is compatible with its role as an intermediate between oxidized and fully reduced flavin. This behaviour most probably is further perturbated by interenzymic interaction with other redox carriers such as the high potential iron protein (HiPIP) [22] and the S_1 center [21,22]. Once oxaloacetate is present the dependence of the flavin radical on redox potential is markedly changed. The magnitude of the signal diminishes and the potential where it attains maximal concentration is shifted by about −200 mV.

It is of interest to note that at physiological potential (potential attained by the succinate fumarate ratio in mitochondira, close to 0 mV) the addition of

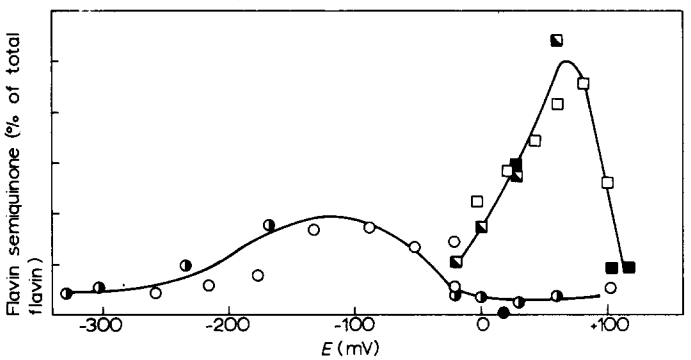


Fig. 2. The dependence of the flavin free radical content of soluble succinate dehydrogenase on the applied redox potential. The enzyme (3.22 mg · ml^{-1}, 3.85 nmol flavin · mg^{-1} protein, 100% reconstitutively active, in 50 mM Tris acetate, pH 7.5, 5 mM succinate) was kept under argon. Deactivation was achieved by addition of 1.66 mM oxaloacetate, enough to cause more than 99.9% deactivation. Oxidation was carried out by addition of 0.5 M furmarate and the reaction mixture was allowed to equilibrate for 5 min at 17°C before sample was removed to the EPR tube. Potentials below −20 mV were achieved by addition of diluted oxygen free solution of $Na_2S_2O_4$. The redox potential was monitored by a combined Pt-silver chloride electrode attached to a Metrohm precision potentiometer. EPR spectra were meaured using a Brucker spectrometer, field modulation frequency 100 kHz, modulation amplitude 0.5 mT, microwave power approx. 1.6 mW, 100 K. The semiquinone concentration was calculated by double integration and comparison with standards of methanol oxidase and flavodoxin free radicals. Circles: (○ ◑ ●) titrations of the non-active enzyme. Squares: (□ ◘ ■) active enzyme. The different signs are for repeating experiments.

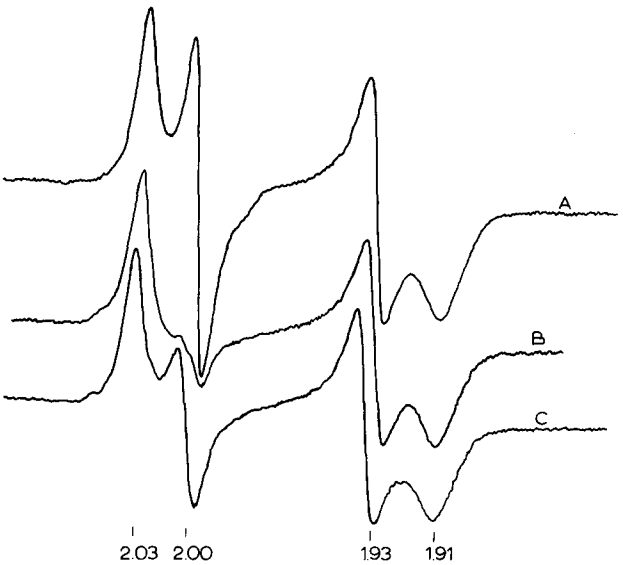


Fig. 3. EPR spectra of soluble succinate dehydrogenase. A, Active enzyme (as in Fig. 2) equilibrated with 5 mM succinate, 0.5 mM fumarate, 50 mM Tris acetate pH 7.5 (E' = +30 mV). B. Non-active enzyme kept under identical conditions in presence of 1.66 mM oxaloacetate (E' = +30 mV). C. Active enzyme reduced by 5 mM succinate ($E' < -30$ mV). EPR spectra were measured as in Fig. 2 but at 15 K, microwave power approx. 2 mM.

oxaloacetate totally quenches the flavin radical signal with little effect on the $g = 1.94$ signal of FeS center (Fig. 3). Upon addition of oxaloacetate, concomitantly with the total loss of the flavin radical, the observed signal of S_1 decreased by about 16%. At +30 mV addition of oxaloacetate abolished the flavin signal with no effect at all on Fe_2S_2 amplitude (compare lines A and B). At either potential the FeS center S_2 (−400 mV [21]) was fully oxidized. Thus we tend to ascribe the big shift in the redox dependence of the flavin radical to the properties of the flavin moiety and not to the FeS centers of the enzyme.

Discussion

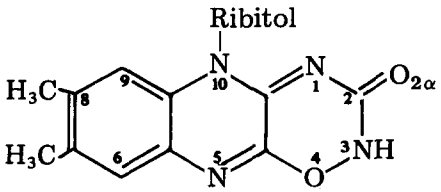
Table I documents that the level of activation of succinate dehydrogenase can be described as a mixture of two populations: one is the active enzyme, with its histidyl flavin at a high redox potential, the other one is the oxaloacetate non active complex. The redox potential of the flavin in this complex is very low, comparable with that of free flavins [9,11].

The two states of the enzyme are also inferred from the redox titrations reported in this study. The redox potential of the flavin in fully active enzyme is compatible with our prediction and so is the 200 mV difference between the redox potential of the two states. Finally, Fig. 3 demonstrates that deactivation does not affect the redox potential of the FeS center S_1 of succinate dehydrogenase.

The precise mechanism which shifts the redox potential of the flavin is still obscure. A possible mechanism is distortion of the flavin from its oxidized

[23] or semiquinone [24] planar forms to the bended configuration of reduced flavin [23]. Theoretical calculations of the stability of oxidized and reduced flavin (Lindner, D.L., Branchoud, B., Doxin, B. and Lipscom, N.W., unpublished results) indicated that the energy associated with distortion of the oxidized flavin is compatible with the measured 200 mV shift of the redox potential.

Another mechanism which accounts for the one-electron activation (Table I) and the 200 mV shift of the redox potential is based on the effect of positive charges (either H^+ or positively charged side chain of the peptide structure) with region 1-2α or N5 of the flavin (see Scheme II).



Scheme II.

A positive charge near the 1/2α position favors a two electron redox reaction with a high redox potential (—9 mV as measured with model compound [25]). Thus if we attribute such interaction, between the apo protein and flavin, in the active enzyme we account both for the high redox potential and the two electron oxidation of succinate to fumarate. Furthermore the close proximity of the positive charge to the flavin can account for the change in chirallity associated with activation as measured by our CD studies [26]. This state of the enzyme is the favoured conformation in the absence of oxaloacetate.

The non-active enzyme is a configuration where the positive charge interacting with 1/2α position is lost. The configuration is stabilized by forming a very tight complex (K_d approx. $2 \cdot 10^{-7}$ M) [8,10,17] with oxaloacetate. Consequently any mechanism leading to displacement of oxaloacetate will remove the constrains and the enzyme will assume its active configuration, a rather slow transition characterized by a high energy of activation (For compilation of experimental values see Ref. 10). The displacement of oxaloacetate from the regulatory site, which is clearly distinct from the substrate binding site (Table II in Refs. 5 and 10), is achieved by variety of activators: succinate, malonate, bromide or one-electron reduction (Table I). A one electron reduction of a free flavin, which is the characteristic behaviour of the flavin in the non active enzyme, leads to protonation at position 5, or attraction of a positive charge to this position. It is proposed that this one electron reduction triggers a conformation change leading to a state characterized by a lower affinity for oxaloacetate K_d approx. $4 \cdot 10^{-4}$ M [10,17]. This coupling between the binding affinity for oxaloacetate and the 1 electron reduction also accounts for the effect of oxaloacetate concentration on the redox potential of the 1 electron reduction (see Table I) [10,17].

Presently we can state that the regulation of succinate dehydrogenase activity operates by modulating the redox potential of the flavin between two states, one is thermodynamically competent to function as electron carrier between substrate and acceptor while the other is not. Better understanding of the mo-

lecular mechanism leading to that effect will assist in understanding the redox reaction of this enzyme.

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