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STRUCTURAL AND KINETIC STUDIES ON THE ACTIVATORS OF SUCCINATE DEHYDROGENASE

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Summary

1. Diverse classes of compounds such as dicarboxylates, pyrophosphates, quinols and nitrophenols are known to activate mitochondrial succinate dehydrogenase (EC 1.3.99.1). Examples in each class — malonate, pyrophosphate, ubiquinol and 2,4-dinitrophenol — are selected for comparative studies on the kinetic constants and structural relationship.

2. The activated forms of the enzyme obtained on preincubating mitochondria with the effectors exhibited Michaelian kinetics and gave double-reciprocal plots which are nearly parallel to that of the basal form. On activation, K_m for the substrate also increased along with V. The effectors activated the enzyme at low concentrations and inhibited, in a competitive fashion, at high concentrations. The binding constant for activation was lower than that for inhibition for each effector.

3. These compounds possess ionizable twin oxygens separated by a distance of 5.5 ± 0.8 Å and having fractional charges in the range of —0.26 to —0.74 e. The common twin-oxygen feature of the substrate and the effectors suggested the presence of corresponding counter charges in the binding domain. The competitive nature of effectors with the substrate for inhibition further indicated the close structural resemblance of the activation and catalytic sites.

Introduction

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase EC 1.3.99.1) from a variety of sources showed the property of activation when mitochondria were pre-incubated with the substrate [1]. A widely differing type of compounds including substrate-competitors have been found to show activation of

Abbreviations: Cl_2Ind, dichlorophenolindophenol; PMS, phenazine methosulphate.

succinate dehydrogenase. These fall into the classes of dicarboxylates, pyrophosphates, quinols and nitrophenols [2]. It had become axiomatic that a competitive inhibitor by implication should activate the enzyme on pre-incubation of mitochondria with lower concentration of the compound [3]. In contrast to the substrate, some effectors gave activated samples which retained the enhanced activity even after repeated washings [2]. While this is indicative of firm binding of these effectors to the enzyme protein — by implication at the "active site" — it is difficult to visualise how the enhanced catalytic activity could be realized subsequently in the assay. In view of the findings that the activation required far less concentration than inhibition, it is possible that all sites were probably not covered by these effectors. A common requirement for the activation is the need for pre-incubation of mitochondria at a specific temperature and with different concentrations of the effectors. Neither oxidation nor any other reaction of the effectors seems to occur. Only a physical, temperature-dependent association of the ligand with the enzyme protein appears to be the underlying feature of this activation phenomenon.

In the present investigation, taking the substrate and one representative example of the four classes of compounds, this activation phenomenon was studied with respect to the binding constants for activation and inhibition and structural relationship of effectors in order to gain insight into the nature of their interaction with the enzyme. By model building it was recognized that these diverse classes of compounds have one common structural feature of possessing two ionizable oxygen atoms spatially separated and the distances are now calculated. An attempt is made to explain the common features, and the small differences, exhibited by the effectors in the activation phenomenon.

Experimental

Succinate dehydrogenase activity was determined spectrophotometrically with phenazine methosulphate (PMS)-2,6-dichlorophenolindophenol (Cl_2Ind) as the electron acceptor system [4]. The reaction mixture contained 100 μmol of potassium phosphate buffer (pH 7.6), 1.2 μmol of KCN (freshly neutralized), 0.75 μmol of $CaCl_2$, and 1—2 mg mitochondrial protein in a volume of 2.7 ml. The reaction was started by successive addition of 0.1 ml each of 50 μmol of succinate, 0.4 μmol of Cl_2Ind and 1% PMS solution and the decrease in A_{660nm} was determined. Reduction of the dye in the absence of succinate was negligible in all cases. The enzyme activity is expressed as nanomoles of dye reduced per min per mg of protein. Pre-incubations were carried out at 37° C, where specified, for the periods indicated. The preincubation mixture contained about 2—5 mg of mitochondrial protein, 100 μmol of potassium phosphate buffer (pH 7.6) and succinate or other compounds at specified concentrations in a total volume of 1 ml. An aliquot of this mixture was used for assaying the enzyme activity at 25° C. In the case of ubiquinol the amount carried into the assay mixture was too small to show any non-enzymatic reduction. Activation was negligible under assay conditions. The activities of enzyme determined after preincubating mitochondrial samples with the buffer alone served as the controls. It is considered appropriate to use mitochondria in this study since the actual regulatory changes occur in membrane-bound state of the enzyme.

Diffusion or permeability barrier is unlikely to be limiting for the accessibility of the effectors since the activation medium is non-isotonic and the membrane structure would have been disrupted during preincubation. Preparation of rat liver mitochondria and other methods are as given in a previous communication [2].

Results

Kinetic constants

The significant feature of the activation phenomenon is that the substrate, succinate, and the competitive inhibitors function as activators of succinate dehydrogenase when mitochondria are preincubated with these compounds before assay. These observations gave indication that the "activation site" and the "catalytic site" may have common structural features. Further relationship of these sites was probed by kinetic studies. Three constants were obtained:

1. K_m for substrate to half-saturate the catalytic site of the enzyme activated by preincubation with each effector;

2. K_a for each effector representing the concentration required to half-saturate the activation site, and

3. K_i the inhibitor constant for each effector.

In the first set of experiments, samples of mitochondria were preincubated with optimum concentration of each effector for obtaining activated enzyme preparations (malonate, 0.01 mM; pyrophosphate, 0.3 mM; ubiquinol, 0.6 mM; 2,4-dinitrophenol, 4.6 mM; succinate, 1.0 mM). Alqiuots of these activated preparations were then used to determine enzyme activity with different concentrations of substrate in the assay medium. Each preparation gave Michaelian kinetics and the data are shown as double-reciprocal plots of $1/v$ versus $1/[S]$ in Fig. 1. For each activated enzyme, a straight line is obtained which is nearly parallel to that of the basal state of the enzyme. This type of activation therefore, does not belong to the known "K" or "V" types. The "apparent K_m" values calculated from Fig. 1, are given in Table I. The activated forms of the enzyme showed higher values of K_m concomitant with increase in V in a synchronous fashion. The experimental results can be fitted by using a multiplication factor $(K_a + [A])/[A]$ for both K_m and V so that the slope K/V is kept constant. The parallel double-reciprocal plots are typical of ping-pong mechanism which requires two substrates and yields two products, the second substrate being added after release of the first product, while the enzyme oscillates between two stable forms during the reaction [5]. In the case of succinate dehydrogenase, neither of the two necessary conditions — the two substrates or the oscillation of two forms of the enzyme during catalysis — is satisfied and therefore the parallel lines can not be explained by this mechanism.

In the second set of experiments, increasing concentrations of the effectors were added in the preincubation medium to obtain activated forms of the enzyme. The enzyme activity was then measured with saturating concentration of substrate in the assay medium. The results are shown in Fig. 2 as double-reciprocal plots of the effector concentration in the preincubation medium versus enzyme activity. The K_a values are calculated by modified Michaelis-Menten equation and shown in Table I. The value of V was nearly the same for

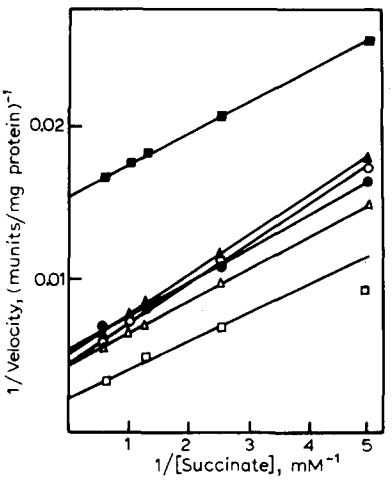


Fig. 1. Activation of succinate dehydrogenase by various effectors. A well-washed, deactivated preparation of rat liver mitochondria was used as the basal enzyme (■). Preincubation of mitochondria was carried out at 37°C for 7 min in the presence of effectors at the concentrations indicated: malonate, 0.01 mM (△); pyrophosphate, 0.3 mM (▲); ubiquinol, 0.6 mM (□); 2,4-dinitrophenol, 4.6 mM (○) and succinate, 1.0 mM (●). Aliquots were used for determining the enzyme activity at 25°C with different concentrations of the substrate. The data are given as double-reciprocal plots and are treated by the method of least square to obtain the lines, with points showing the experimental values.

effectors, malonate, pyrophosphate, 2,4-dinitrophenol and succinate, but was 2-fold higher for ubiquinol. The efficiency of activators on the basis of concentration for maximal activation is in the following decreasing order: malonate, pyrophosphate, ubiquinol, succinate and 2,4-dinitrophenol. The K_a value for each effector is lower than the K_m value for the substrate in all the activated forms of the enzyme.

In the third set of experiments, the sample of mitochondria was fully activated by preincubating with 5 mM succinate. The enzyme activity was determined with different concentrations of substrate in presence of effectors at high concentrations sufficient to show inhibition (malonate, 0.17 mM; pyrophosphate, 0.03 mM; 2,4-dinitrophenol, 1.67 mM). The results are shown in Fig. 3 as double-reciprocal plots of $1/v$ versus $1/[S]$ for each effector. The inhibition pattern is of the classical competitive type with a common intercept on the ordinate. The inhibitor constant, K_i, was calculated from the relationship: "apparent K_m" = K_m (K_i + [I])/K_i where [I] represents the concentration of the effector used in the assay medium. The values of K_i for each effector and "apparent K_m" for the substrate are given in Table I. It can be seen that "apparent K_m" values increased in presence of the effectors at inhibitor concentrations. The values of K_i are higher than the corresponding values of K_a for each effector.

Structural relationship

The common structural feature in the four classes of activators (dicarboxylates, pyrophosphates, quinols and nitrophenols) is the presence of two oxygen atoms separated by a distance upto about 7 Å as judged by Coutauld's atomic

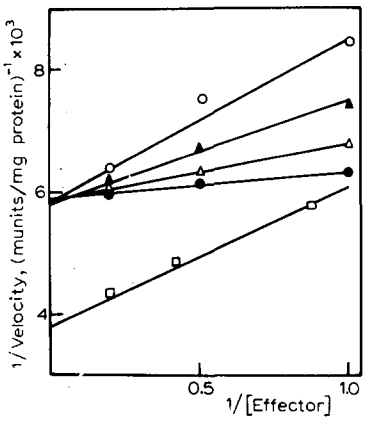
TABLE I

COMPARATIVE STUDY OF THE PROPERTIES OF EFFECTORS

Activator used:	Class of compound				
	Dicarboxylates (malonate)	Pyrophosphates (pyrophosphate)	Quinols (ubiquinol)	Nitrophenols (2,4-dinitro-phenol)	Substrate (succinate)
I. Structural features					
1. Distance between twin oxygens (Å)	4.68	4.75	5.50	6.20	6.02
2. Fractional charges on twin oxygens (e)					
O_1	−0.62	−0.37	−0.73	−0.26	−0.62
O_2	−0.62	−0.37	−0.73	−0.73	−0.67
II. Conditions for activation and reversal *					
1. Preincubation time for maximum activation (min):					
at 30° C	6	2	—	1	—
at 37° C	2	fast	2	fast	7
2. Reversibility of activation on washing mitochondria with sucrose					
Without succinate	stable	partial	stable	partial	reversed
With added succinate in washing medium	reversed	reversed	stable	reversed	reversed
III. Kinetic constants					
1. Apparent K_m ($\times 10^{-5}$ M) **					
activated enzyme, $K_{m(a)}$	46	48	81	57	42
Inhibited enzyme, $K_{m(i)}$	360	80	—	110	—
$K_{m(i)}/K_{m(a)}$	7.8	1.7	—	2.0	—
2. Binding constants of effector ($\times 10^{-5}$ M)					
For activation, K_a	0.14	2.9	6	46	8
For inhibition, K_i	2.7	5.0	—	150	—
K_i/K_a	19.3	1.7	—	3.2	—
3. V (nmol/min/mg of protein)	210	222	413	211	208

* Data under II are collected from Susheela and Ramasarma [2,3].

** Effectors were added at concentrations needed for maximal activation or about 50% inhibition; without any conditions: $K_m = 13 \cdot 10^{-5}$ M, $V = 63$ nmol/min/mg protein.



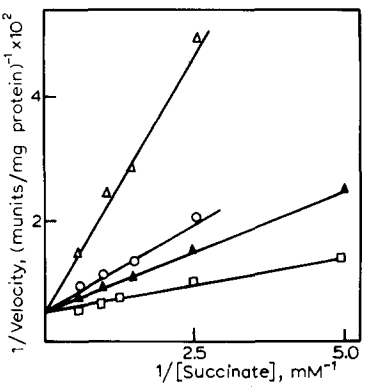


Fig. 2. Binding constants of effectors for activation (K_a). Samples of mitochondria were preincubated with different concentrations of effectors and aliquots from these mixtures were used to determine the succinate dehydrogenase activity. The data are represented as double-reciprocal plots of concentration of effector in the preincubation mixture versus activity of the enzyme. Binding constants, K_a, are calculated by modification of Michaelis-Menten equation and given in Table I. Malonate (△); pyrophosphate (▲), ubiquinol (□), 2,4-dinitrophenol (○) and succinate (●).

Fig. 3. Binding constants of effectors for inhibition (K_i). Mitochondria were first preincubated with 5 mM succinate to activate the succinate dehydrogenase. The enzyme activity was then determined in presence of effectors added in assay medium at concentrations which showed inhibition. Binding constants (K_i) are calculated from the relationship: "apparent K_m" = K_m (K_i + [I])/[I], and given in Table I. Basal (■), malonate, 0.17 mM (△), pyrophosphate, 0.03 mM (▲) and 2,4-dinitrophenol, 1.67 mM (○).

model and capable of ionization at physiological pH [2]. It is therefore considered desirable to have a comparative study employing precise data from literature on the physical parameters of distance between the twin oxygen atoms and the fractional charges they possess. Using X-ray crystallographic data of these compounds or their derivatives, longest O-O distance, represented vertically in Fig. 4 have been calculated based on the fractional atomic coordinates as reported: malonate [6], pyrophosphate [7], quinol [8], nitrophenol [9] and succinate [10]. The distances vary from 4.7 Å for malonate to 6.2 Å for 2,4-dinitrophenol (Table I). It is interesting to note that other compounds which are effective as activators also gave O-O distance in the range of 5—6 Å. Methyl malonate was less efficient than malonate and its dissociation constant is also known to be less than that of malonate. In the case of 2-oxoglutarate, a dicarboxylate ineffective for activation, the ionization O-O distance cannot be brought to less than 6.5 Å between the two carboxylate oxygens.

In view of the possible requirement of two ionizable oxygens in the ligands, the other parameter of importance is the fractional charge associated with the oxygen atoms. The π-charges of the oxygens of nitrophenol and hydroquinone were calculated from Huckel's molecular orbital methods [11]. The charges of oxygens in dicarboxylates were obtained by analogy with the charges given for amino acids [12]. In the case of pyrophosphate, the charges were obtained from a knowledge of the bond moments given for P-O bond [13]. The values are approximations and are expected to suffice for qualitative comparisons attempted here. The fractional charge ranged from —0.26 e to —0.73 e on

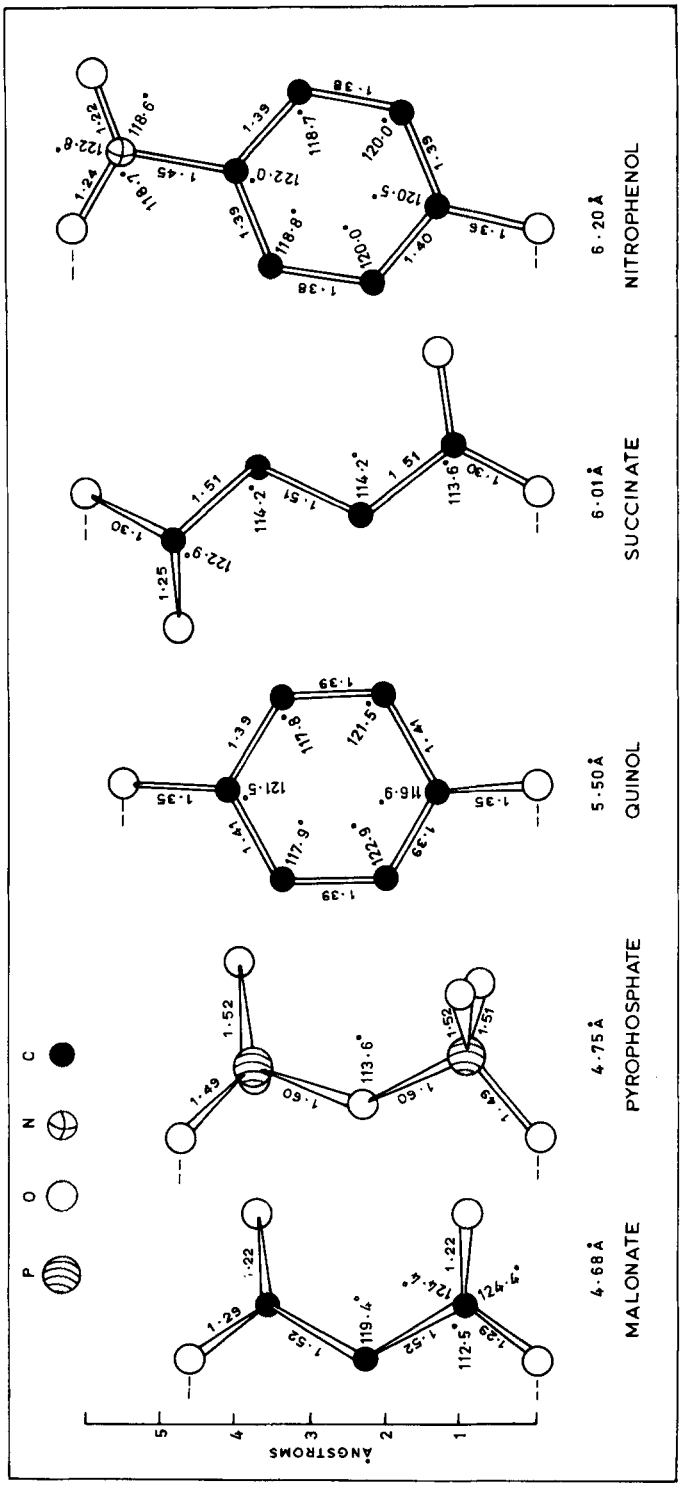


Fig. 4. Three dimensional view of the effectors depicting the twin oionizable oxygens (O_1—O_2) arranged vertically in the plane along the scale indicated. The O-O distances are shown for each effector. Hydrogen atoms are omitted.

individual oxygens (Table I). Ubiquinol, having oxygens with maximal fractional charge, showed maximum activation which could not be reversed in the washing procedure.

Discussion

The functional meaning of subunit structure of polymeric enzymes became apparent with discovery of allosteric properties manifesting cooperative kinetics. But enzymes showing classical Michaelian-type kinetics and having oligomeric structures are numerous and each subunit in such an enzyme is presumed to act independently. This, however, fails to give any clue for the need of subunit structure. Observations that *low* concentrations of the substrate, or its structural analogues, can activate enzymes have implications in regulation of overall metabolic activity, especially if a dimer becomes the functional unit, with one site acting as the "activation site", and the second one assuming the role of the "catalytic site". Such an enzyme possesses a subtle regulatory potential whereby its active form can be rapidly generated on arrival of increased amounts of the substrate and it reverts to a dormant or low-activity form after the exhaustion of the substrate, without recourse to energetically expensive protein turnover. The example of mitochondrial succinate dehydrogenase offers a possibility of studying such an activation phenomenon.

The clue obtained from the identification of two juxtaposed negatively charged oxygens is the probable presence of positively charged counter ions in the concerned binding domain on the protein. Since activation proceeded at pH 7.6, these could be provided by nitrogens of lysine or arginine. For example, lysine is known to bind to carboxyl group of 2,3-diphosphoglycerate in haemoglobin (Lys 82, β-chain) [14], to intramolecular carboxyl anion in chymotryspin (Lys 107 and Asp 245) [15] and the substrate-phosphate oxygen (Lys 41) in ribonuclease [16]. It is instructive to point out that in the case of horse liver alcohol dehydrogenase, blocking of charged lysines by methylation resulted in severalfold activation [17]. Since the average distance of twin oxygens is about 5.5 ± 0.8 Å, it is tempting to speculate that the counter charges may be located with the amino acid residues, placed at n and $n + 4$ positions, on a α-helix which, with a pitch of 5.39 Å, can provide such a distance. The flexibility of the side-chain, like that of lysine, could account for the variable distance permitted in the compounds known to be activators. Application of the hypothesis of existence of counter charges might hopefully resolve the diverse nature of activators.

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