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INTERACTION OF SUCCINATE UBIQUINONE REDUCTASE WITH SULPHATE ION

*ZABTA M.CHAUDHRY[a], MUHAMMAD AFZAL KHAN[a] and ANDREI D. VINOGRADOV[b]

[a]*Department of Pharmacy, B. Z. University, Multan, Pakistan.*
[b]*Department of Biochemistry, Faculty of Biology Moscow State University, Moscow.*

ABSTRACT

A new type of slow change of succinate Ubiquinone reductase (EC 1.3.99), activity which is induced by sulphate ion is described. After preincubation of submitochondrial particles or succinate uniquinone reductase with sulphate both preparations catalyze succinate: phenazine mcthosulphate reductase reaction with a significant lag. When added to the assay medium sulphate ion induces biphasic time-dependent competitive inhibition of the enzyme. The sulphate-induced inhibition is apparently due to a rapid interaction of the anion with an active site of the enzyme which is followed by a slow pH-dependent (pK = 7.2) transformation of the enzyme inhibitor complex. pH profiles of the overall succinate ubiqunone reductase reaction and of equilibrium between fast and slow enzyme-sulphate complexes suggest that the same protolytic equilibrium step is involved in the formation of an active intermediate and an inactive enzyme-sulphate complex.

Introduction

The specific complex formation between succinate ubiquinone reductase and substrate anions (or competitive inhibitors) has been demonstrated in numerous studies with the use of classical steadyslate kinetics or by direct spectrophotometric approaches. The quilibrium constants and individual rate constants for the formation of the complexes between succinate ubiquinone reductase and some specific ligands [succinate (Zeylemaker, WP. et al 1969; Vinogradov, A.D. et al 1979), malonate (Thom, M.B. 1953; Dervartenian, D.V. et al 1964; Vinogradov, A.D. et al 1976; Schreiber, G. et al 1978), oxaloacetate (Pardee, A.B. et al 1948; Wojtczak, L. et al 1969; Zeylemaker, W.P. et al 1969; Zimakova, N.I. et al 1970; Brodie, J.D. et al 1970; Gutman, M. 1977)] have been measured. The satisfactory agreement between the measured values of equilibrium constants and those calculated from the individual rate constants using the Law of Mass Action have been obtained for those ligands.

The affinities of some ligands such as malonate or oxaloaceiate for the enzyme are very high; consequently the rates of dissociation of the enzyme-inhibitor complexes are low and the time needed to establish an equilibrium

* For correspondence

between the enzyme, substrate and inhibitors may be as high as several seconds [(Coles, C.J. et al 1977) or minutes (Zimakova, N.I. et a11970; Ackrell, B.A.C. et al1974; Gutman, M. 1977)]. The slow dis- sociation of the inhibitors from the active site causes a slow change of the enzyme activ- ity and leads to an apparent 'activation' of succinate ubiquinone reductase the phenome- non which was first discovered in the pioneering work of Kearney and co-workers (Kearney, E.B. et al1955; Kearney, E.B. 1957) and later widely studied in several laboratories (Thorn, M.B. 1962; Kimura, T. et all963; Wojtczak, L. 1969; Zimakova, N.I. et al 1970; Ackrell, B.A.C. et a1 1974; Gutman, M. 1977; Gutman, M. 1978).

It has been shown that some inorganic anions specifically interact with succinate I ubiquinone reductase or fumarate reductase, acting as 'activators' of mammalian and bacterial enzymes (Kearney, E.B. et a11974; Gutman, M. 1976; Robinson, J.J. et al 1981) and as the inhibitors of the bovine heart enzyme (Bonomi, F. et al 1981). In this paper we present evidence that sulphate ion specifically interact with the active site of succinate ubiquinone reductase with relatively low.

Materials and Methods

Submitochondrial particles and the re-constitutively active succinate ubiquinone re- ductase were prepared from bovine heart muscle as described (Tushurashvili, P.R. et al 1985). To remove tightly bound oxaloacetate the particles (1 mg protein per ml) were in- cubated at 25°C for 3 h in 20 mM phosphate buffer (pH 7.8) in the presence of 10 mM potassium malonate, cooled and sedimented at 105000 x g. The sediments were washed three times with 0.25 M sucrose by repeated centrifugation; the final pellets were sus- pended in 0.25 M sucrose and stored in liquid nitrogen. Two criterial were used for the absence of bound oxaloacetate and malonate: (i) no detectable lag was observed when succinate-phenazine methosulphate (PMS) reductase reaction was started by the addition of particles to the assay mixture, and (ii) prolonged preincubation of the preparation in the presence of succinate at 30°C did not increase the succinate ubiquinone reductase ac- tivity. succinate ubiquinone reductase activity was measured in the standard, 2 ml assay mixture comprising 20 mM succinate, 0.1 mM EDT A, 20 mM Hepes (pH 7.8), 1 mM cyanide (potassium salts), 2 mM PMS and 30 mM Wurster's blue or Mitochoundria and 30 JJM Wurster's blue and 5 ~ Q2 for succinate ubiquinone reductase. The reaction was started by the addition of the proper amount of the enzyme preparation. Protein was de- termined by the biuret method (Gornall, A.D. et al 1949).

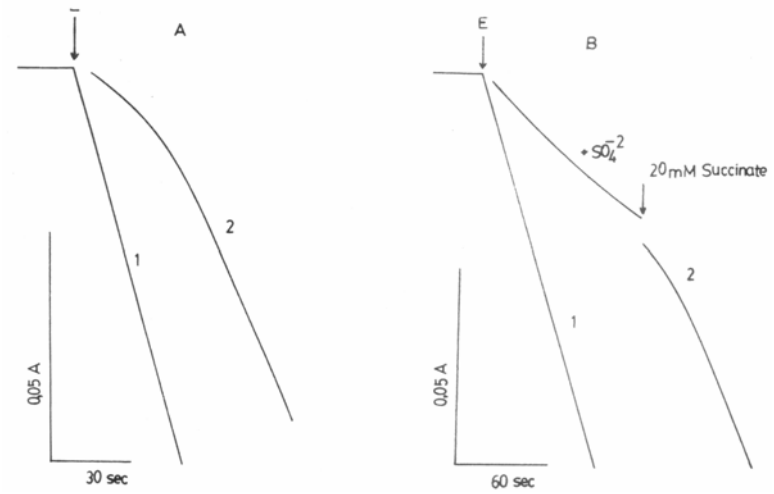


Fig. 1: The effect of sulphate on the kinetics of succinate oxidation. (A) Submitochondrial particles (0.5 mg/ml) were incubated for 20 min at 25°C in the mumre comprising 20 mM Hepes (pH 7.8/0.1 mM EDTA (potassium salts) in the presence (curve 2) or in the absence (curve 1) of 50mM potassium sulphate: the reaction was started by the addition of 20 µl of the mixture to the assay medium. (B) Submimchondrial panicles (0.5 mg/ml) were incubated as before without potassium sulphate and the ruction was suited by the addition of 12.5 µl of the mixture to the assay medium containing 1 mM succinate and 5 mM potassium sulphate (where indicated).

Results and Discussion

The effect of sulphate on the kinetics of the succinate-phenazine methosulphate re- ductase reaction catalyzed by submitochondrial particles is shown in Fig. 1. When the enzyme assay is started by the particles preincubated in the presence of 50 mM ~SO 4 a significant lag in their succinate-phenazine methosulphate reductase activity is evident (Fig. lA, curve 2) and the rate of the reaction becomes constant and equal to that of the control trace after approx. 3 min. The substitution of K_2SO_4 in the preincubation mixture does not change the pattern of DCIP reduction, whereas the particles preincubated with PO_4^{-2}, Cl^{-1}, Br^{-1}, F^{-1}, CIO_4^{-1}, $NO3^{-1}$ potassium salts) catalyze the reaction without any detectable lag. When the instant rates of the reaction (v_t) represented by the curves with a lag were determined with the use of a higher time-scale resolution (chart speed 12 cm/ min) and the values of in (v_t-v_t-v_t) were plotted as a function of the reaction time (t) the resulting straight line with a slope (first-order rate constant) equal to 1.4 min^{-1} intersects the ordinate at the point corresponding to 20% of the control rate (no sulphate in the pre-incubation medium) (Fig. 2). Neither initial rate nor 'activation' pattern were changed when sulphate concentration in the preincubation medium was increased up to 0.2 M; at a given value of pH. It should be noted that since activation of the sulphate-inhibited en-zyme is rather fast the precision of our first-order rate constant determination is about 20%.

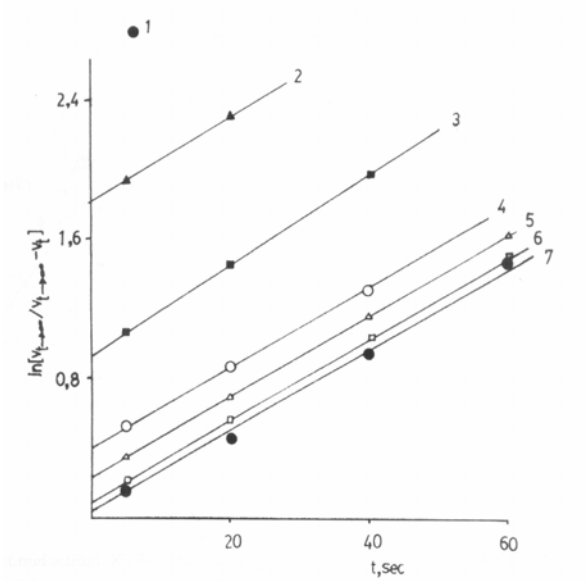


Fig. 2: The effect of pH on the inactivation of succinate by sulphate ion. Sulanitochondrial panicles (0.5 mg/ml) were incubated for 20 min at 25°C in the mixture comprising 20 mM Hepes, 0.1 mM Hepes, 0.1 mM EDTA and potassium sulphate at the different pH, 1-6,0; 2-6,5; 3-7,0: 4-7,5; 5-8,0; 6-8,5: 7-9,0. The reaction was started by the addition of 20 at of the mixture to the assay for measuring activity. v_t was calculated as (Fig. 1A, curve 2) and v_t, at the same condition but in the absence of K_2SO_4.

The presence of sulphate in the assay mixture also affects the Kinetics of succinate oxidation (Fig. 1B). In this case the initial rate of the reaction is decreased and the time-dependent increase in inhibition is evident, this time-dependent inhibition is evidently not due to an irreversible inactivation of the enzyme, since succinate added in excess (20 mM) restores the activity up to the control level (no sulphate in the assay mixture).

Both instant and time-dependent effects of sulphate obey a simple competitive inhibition pattern, with K_i values determined from Dixon plots of 6 mM (K_i^{fast}) and 2 mM (K_i^{slow}) respectively. (Fig. 3).

It was also found that qualitatively the same pattern of sulphate ion effects is observed when active succinate ubiquinone reductase is used as an enzyme preparation in the presence of either PMS or Wurster's Blue and Q_2 as artificial electron acceptors. This finding suggests that the target of the sulphate effect is the flavoprotein, and the modulation of the enzyme activity is not directed to any memberane-bound component of the succinate ubiquinone reducatase system.

The sulphate-induced slowly reversible inhibition of succinate ubiquinone reductase is strongly pH-dependent. Fig. 4 (curve 2) demonstrates the dependence of inhibition of the initial rate on the pH of the preincubation

medium. The experimental points closely fit the 'heretical curve for a protonation of monobasic acid with a pK value of 7.2. It seemed of interest to compare the pH dependence of the enzyme inhibition by sulphate with that of the maximal activity of succinate ubiquinea- reductase. Fig. 4 (curve 1) demonstrates such a dependence. The remarkable coincidence of pK values for the sulphate-induced slowly reversible inhibition and for the maximal enzyme activity is evident and suggests that ionization of the same group of the enzyme is a prerequisite for both processes. Although the degree of inhibition by sulphate is strongly pH-dependent, the first-order raw constant for activation of the inhibited enzyme was found to be constant (1.4 min^{-1}) within the pH interval from 6.5 to 8.5.

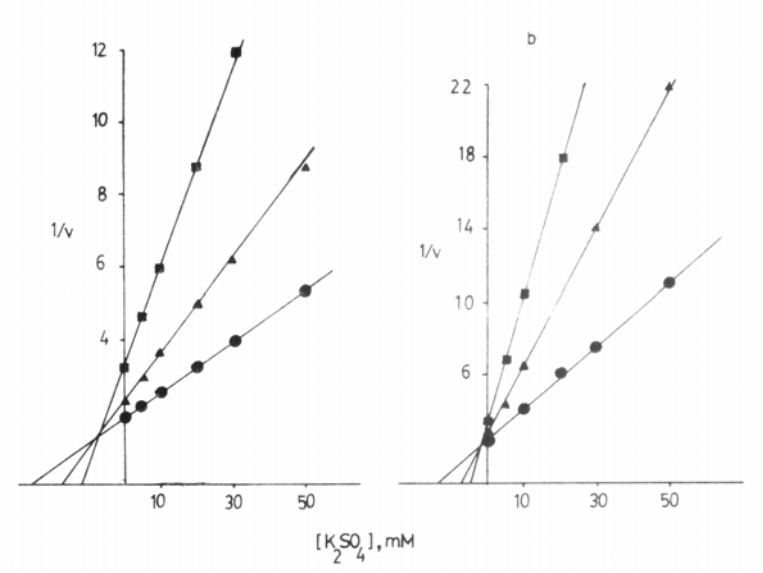
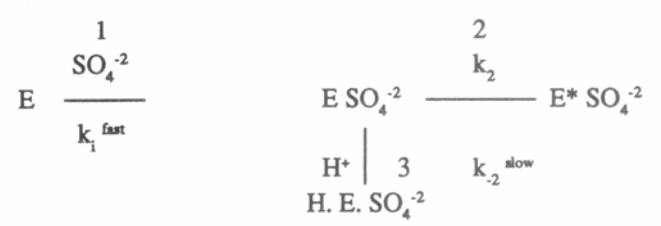


Fig. 3: Kinetics of succinae dehydrogenase inhibition by sulphate ion. Activity was measured in the as-say containing succinate (conc. in μM) 1-50; 2-100; 3-200 and potassium sulphate conc. is shown at ebcissa. The reunion was suited by the addition of 20 μl of the mixture (0.5 mg/ml). a-Right from the start, b-after the 3 min from starting (as fig. 1b).

The simplest hypothesis which accounts for the experimental observations reported here may be represented as follows:



The formation of $E.SO_4^{-2}$ complex where sulphate is bound to the active site is evident from the simple competitive inhibition of the enzyme by sulphate. The slow isomerization of the primary enzyme-inhibitor complex (reaction 2) is supported by the slow activation of the enzyme preincubated with saturating concentrations of sulphate ($k = 1.4\ min^{-1}$) and by the time-dependent increase of the competitive inhibition (Fig. 1).

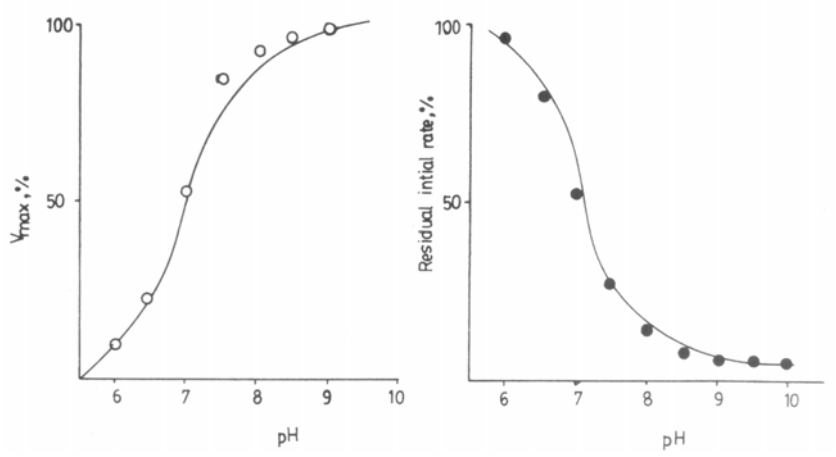


Fig. 4: The pH dependences of the maximal rate of succinate oxidation and sulphate-induced inhibition. (1) The enzyme was assayed in the standard mixture containing 40mM poussiwn succinate and 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 or 1 mM Wurster's Blue; the maximal activity was obtained by extrapolati to infinite acceptor concentration at each pH, 100% activity corresponds to 1.2 µ mol of succinate oxdized per min per mg of protein. (2) The inhibition of the initial rate of succinate oxidation by 50 mM potassium sulphate (see Fig. 1A) was determined as a function of the pH values of the preincubate mixture. The solid lines represent the theoretically calculated pH functions for dissociation (curve 1) or protonation (curve 2) or monobasic with pK_a value of 6.8 and 7.2, respectively.

The value of K may be calculated assuming that the equilibrium constant for reaction2 equal 3 (the ratio between K_i^{fast} and K_i^{slow}) and using the experimentally determined value of K; the calculation gives $k_2 = 5.2\ min^{-1}$. The dependence of slow isomerization on pH in the presence of saturating sulphate (approx. 10K) and the independence of K_{-2} of pH show that the protonated 'rapid' enzyme-sulphate complex cannot be transformed into the slow $E^*.SO_4^{-2}$ complex.

The low rate of reaction 2 indicates that strong conformational changes are involved in the process of isomerization (Jenks, W.P. 1969). It is noteworthy that the activation of oxaloacelate – and malonate-inhibited enzyme is also slow and highly temperature-dependent (Ackrell, B.A.C. et al 1974; Coles, CJ. et al 1977), thus indicating significant conformational change during the formation of enzyme inhibitor complexes. The remark-able similarity of pH-dependence of reaction 2 to that of the maximal rate of succinate oxidation opens an interesting

possibility that the sequence of reactions 1 and 2 might be considered as a model for the initial step of succinate oxidation. It may be speculated that when the primary enzyme-substrate complex is formed during catalysis, the protolytic equilibrium (reaction 3) also exists and the high activation energy 2 is compensated by an oxidizing power of the redox components of the enzyme. The specificity of sulphate in the formation of the slow complex as compared with other inorganic suggests that either the primary complexes with those anions cannot undergo isomerization or the isomerization occurs very rapidly.

One practical aspect of the sulphate-induced slow transformation of the enzyme merits special emphasis. The values of substrate or inhibitor binding constants for succinate ubiquinone reductase reported in the literature vary over a wide range. We believe that one, perhaps the most significant, reason for such variations is the difference in ionic composition and pH values of the buffers used by different investigators. The existence of the slow dissociating complex between enzyme and sulphate anion suggests that some data on quantitative aspects of succinate ubiquinone reductae Kinetics and site-directed inhibition must be re evaluated.

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