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THE INACTIVATION OF SUCCINATE DEHYDROGENASE BY BROMOPYRUVATE

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SUMMARY

1. Bromopyruvate has been found to be a potent irreversible inhibitor of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1). While the kinetics of inactivation show a saturation effect, they do not fit the simple model for an active-site-directed irreversible inhibitor. Instead, a concentration dependent plateau is observed. A similar phenomenon occurs when *N*-ethylmaleimide is the alkylating agent. Reversible inhibitors and substrates protect succinate dehydrogenase from inactivation by bromopyruvate and *N*-ethylmaleimide. Both reagents have similar relative reactivities toward the enzyme and 2-nitro-5-thiolbenzoic acid dianion.

2. The absorption and dithionite bleaching spectra are not greatly affected by the reaction of enzyme with bromopyruvate, but the ability of succinate to bleach is lost. A decrease in hydrogen exchange capacity parallels the decrease in assay activity.

INTRODUCTION

In spite of varied approaches to the problem, comparatively little is understood about the mechanism of catalysis of succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1). Both covalently bound flavin and at least some of the non-heme iron residues have been implicated as prosthetic groups[1,2]. Apart from the isolation of flavin proteins[3,4] and the more recent separation of a purified succinate dehydrogenase preparation into two peptides[5,6], there is also little information about the structure of the enzyme. The size and conformational requirements of the active site have been studied through the limited number of substrates and reversible inhibitors which can interact with the enzyme[7,8].

Succinate dehydrogenase is a classical "sulfhydryl" enzyme which is inactivated rapidly by mercurials and progressively by certain disulfides, arsenicals, and common alkylating agents such as *N*-ethylmaleimide and iodoacetate[9]. Succinate or competi-

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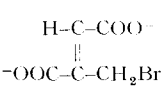
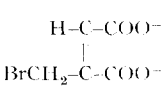
tive inhibitors, such as malonate or oxaloacetate, are observed generally to retard the rates of inactivation.

In the present study, alkylating agents with structures similar to substrates or competitive inhibitors were chosen for preliminary investigation in an effort to discover an active-site-directed irreversible inhibitor or, at least, to introduce an element of selectivity into the inactivating agent. The selection of compounds was influenced by the finding of HELLERMAN *et al.*[10] (see also WEBB[11] and references therein), that binding at the active site of succinate dehydrogenase is not limited to compounds possessing two carboxylic acid groups; the authors postulated that oxaloacetic acid monoethyl ester interacts *via* the enolate. Recently ZEYLEMAKER *et al.*[12], also reported K_d values for several monocarboxylic acids, the most tightly bound of which contained α-hydroxyl groups. The compounds investigated in the present study were bromopyruvate (I), chlorolevulinate (II), the fumarate analog bromomesaconate (III), and the maleate analog bromocitraconate (IV). One of these, bromopyruvate, proved to be an especially potent inactivator of the enzyme. The nature of the reaction was explored. For comparison, two typical sulfhydryl reagents with greatly differing reactivities were used: iodoacetate (V), a slow reagent, and *N*-ethylmaleimide (VI), a relatively rapid reagent.

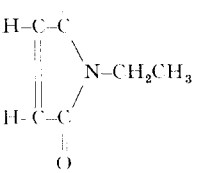
(I) $BrCH_2COCOO^-$

(II) $ClCH_2COCH_2CH_2COO^-$

(III)


(IV)


(V) ICH_2COO^-

(VI)


MATERIALS AND METHODS

Sodium malonate (Eastman), sodium succinate (Eastman), *N*-ethylmaleimide (Mann) and oxaloacetic acid (Calbiochem) were used as received. Iodoacetic acid (Mann) was recrystallized from methanol–light petroleum (m.p. 75–82°).

Chlorolevulinic acid (5-chloro-4-oxopentanoic acid) was prepared by the method of RAPPE[13] from β-carbomethyoxypropionyl chloride, which was made by addition of phosphorous pentachloride to succinic acid monoethyl ester[14] in ether[15] (m.p. 68–69.5°).

Bromopyruvic acid (Eastman (m.p. 52–54°) and Nutritional Biochemical (m.p. 58–59°)) was used as received or after recrystallization (m.p. 58–59°). Bromo-[2-^{14}C]pyruvic acid was prepared as described by MELOCHE[16] from sodium [2-^{14}C]-pyruvate (New England Nuclear, 4.83 mC/mmole), redistilled pyruvic acid (Eastman), and bromine. The reaction mixture was stored frozen. Aliquots were treated as described[16] and finally taken up in 0.1 M potassium phosphate, pH 7.0, for use in the alkylation experiments. The concentration of ketoacid present was estimated as the

semicarbazone using sodium pyruvate as standard[17]; this was assumed to represent the concentration of bromopyruvate. Radioactivity was measured in BRAY's[18] solution. Specific activities ranged between $1.1 \cdot 10^9$ and $1.5 \cdot 10^9$ counts/min per mole. Hydroxypyruvate was prepared by alkaline hydrolysis of bromopyruvate.

Bromomesaconic acid (m.p. 180–182°) and bromocitraconic anhydride were gifts from R. A. Laursen. Bromocitraconic acid was made by the addition of the anhydride to water. The resulting acidic solution was stable for more than one day. Bromomesaconate, the most labile of the compounds in aqueous solution, has a half life of about 15 min in 10 mM phosphate buffer, pH 7.3[19].

Protein-bound flavin was estimated from the magnitude of the sodium dithionite difference spectrum at 465 nm using the extinction coefficient $2 \cdot 10^4$ A (M flavin)$^{-1}$ (ref. 20). Hydrolytic release of flavin peptides by proteolytic enzymes prior to spectrophotometric analysis provided an additional measurement of peptide-bound flavin[21]. The concentrations of succinate dehydrogenase expressed in this paper refer to the concentrations of peptide-bound flavin.

Succinate dehydrogenase activity was assayed spectrophotometrically at 600 nm by a slight modification of the procedure of ARRIGONI AND SINGER[22]. Specific activity was calculated using an extinction coefficient of 21 000 for 2,6-dichlorophenolindophenol at 600 nm[23]. Turnover numbers of about 3000 moles succinate · (mole peptide-bound flavin)$^{-1}$ · min^{-1} were observed at 22° and apply to previously activated enzyme.

Starch–agar slabs for electrophoresis (25 mA, 200 V, 0°, 1 h) were poured from a hot solution of 4.5 g hydrolyzed starch (Connaught) and 3.0 g Ionagar 2 in 300 ml 0.02 M Vernol buffer, 1 mM EDTA, pH 8.6. Buffer chambers contained 0.06 M Vernol buffer and 1 mM EDTA. The stain for succinate dehydrogenase contained 50 mM potassium phosphate (pH 7.4), 24 mM sodium succinate, 1 mM EDTA, 0.3 mg/ml *p*-nitrotetrazolium blue and 0.7 mg/ml phenazine methosulfate. The stain for protein was 1% amido black in methanol–glacial acetic acid–water (5:2:5, by vol.).

Purification of succinate dehydrogenase

Purification of soluble enzyme from bovine heart was carried through the first ammonium sulfate fractionation (25–46% saturation based on 100% as 706 g/liter)[24,25]. Starch–agar electrophoresis revealed the presence of acidic and basic contaminants which the following procedures eliminated.

The ammonium sulfate precipitate was dissolved in 0.01 M Tris–HCl, 1 mM EDTA, pH 7.7 buffer and applied to an ascending Sephadex G-150 column equilibrated with the same buffer. The bulk of the succinate dehydrogenase activity was eluted soon after the void volume of the column as expected for an enzyme of mol. wt. 150 000–200 000[24,26]. Approx. 80% of the total activity was pooled although 98% could be accounted for, indicating that little enzyme activity was lost by the procedure. The pooled material was then applied to a DEAE 11 cellulose column equilibrated with the same buffer as above and eluted with a linear gradient in NaCl (0.01 M Tris–HCl, 0.04 M NaCl–0.01 M Tris–HCl, 0.45 M NaCl) containing 1 mM EDTA, pH 7.7. It was possible to account for nearly 100% of both protein and activity in the eluant. The major activity peak was used for quantitative studies.

Purity estimates routinely ranged from 25–35% based on flavin:protein[27] ratios and a molecular weight of 150 000–200 000[1,24,26]. Electrophoresis in starch-agar revealed one and sometimes two diffuse protein bands coincident with succinate

dehydrogenase activity. Upon disc electrophoresis[28] (5.5% polyacrylamide, 5°) protein bands devoid of activity were observed in addition to one or two diffuse succinate dehydrogenase bands.

Reaction with alkylating agents

Succinate dehydrogenase was generally incubated at 38° in 50 mM phosphate, 1 mM EDTA, pH 7.0 to activate[29] the enzyme. The activity increased several fold and reached a maximum after 15–30 min. Alternatively the enzyme was activated by incubation with malonate or succinate, followed in some cases by dialysis.

A small volume of a concentrated solution of the alkylating agent was added to the enzyme solution to initiate the reaction; 5-μl aliquots were withdrawn at various times and diluted to 2.3 ml in order to stop the reaction. The diluent contained the ingredients for the activity assay except for the redox dyes and, in some cases, succinate. Assays were initiated by addition of the dyes or of succinate and the dyes. Since the alkylating agents experienced a final dilution of 600-fold, they had no detectable effect in the assay solution.

The reactivity of the several alkylating agents toward 2-nitro-5-thiolbenzoic acid dianion was studied by the spectrophotometric method of LEE AND MCELROY[30].

RESULTS

Efficiency of alkylating agents

As illustrated in Figs. 1 and 3, 1 mM bromopyruvate rapidly inactivates 0.01 mM succinate dehydrogenase with half-times of 2 to 10 min at 22°. Table I shows that *N*-ethylmaleimide exhibits a similar rate while little or no inactivation is observed within 45 min for bromomesaconate, bromocitraconate, chlorolevulinate, and iodoacetate under comparable conditions. As indicated in Table I, inactivation by bromocitraconate becomes clearly evident at 10 mM, whereas the rates of inactivation of the remaining three halide derivatives are still slow. The rank order of reactivity toward the enzyme is (bromopyruvate, *N*-ethylmaleimide) $\gg$ bromocitraconate $>$ (iodoacetate,

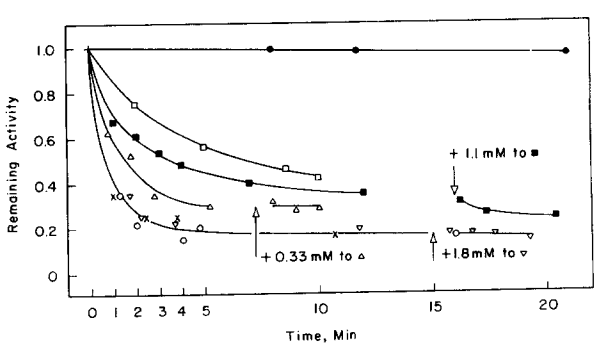


Fig. 1. The effect of bromopyruvate concentration on the rate and extent of inactivation of succinate dehydrogenase. The reaction mixtures (50 mM phosphate buffer, pH 7.0, 22°) contained 0.01 mM succinate dehydrogenase previously activated in phosphate buffer at 38° and bromopyruvate as indicated. ▽, 1.8 mM; ○, 1 mM; ×, 0.5 mM; △, 0.33 mM; ■, 0.22 mM; □, 0.14 mM; ●, bromopyruvate omitted. The arrows mark the further addition of bromopyruvate.

TABLE I

EFFECTIVENESS OF ALKYLATING AGENTS IN INACTIVATING SUCCINATE DEHYDROGENASE

The reaction mixture (22°) contained alkylating agent, 50 mM potassium phosphate buffer (pH 7.0), and 0.01 mM succinate dehydrogenase flavin. The enzyme was activated by pretreatment with malonate (4 mM, 25°, 1 h) followed by dialysis.

Compound added	*Initial concn. (mM)*	*Fraction of initial enzyme activity remaining after 45 min*
None	—	1.05
Bromopyruvate	1	0.15
N-Ethylmaleimide	1	0.18
Bromocitraconate	1	0.98
	10	0.55
Iodoacetate	1	0.94
	10	0.79
Bromomesaconate	1	0.97
	10	0.85
Chlorolevulinate	1	1.04
	10	0.90

bromomesaconate, chlorolevulinate). Inactivation by *p*-hydroxymercuribenzoate is essentially instantaneous under the conditions of the assay.

Table II indicates that the relative reactivities of these alkylating agents toward the model thiol 2-nitro-5-thiolbenzoic acid dianion[30,31] are essentially the same as those toward succinate dehydrogenase. The second order rate constants are similar also. Comparing bromopyruvate for example, k is $2 \cdot 10^3$ $M^{-1} \cdot min^{-1}$ for reaction with

TABLE II

REACTIVITY OF ALKYLATING AGENTS TOWARD 2-NITRO-5-THIOLBENZOIC ACID DIANION

The reaction mixture contained approximately 0.05 mM of the dianion, produced by 0.025 mM dithiothreitol from 10 mM dithiobis(2-nitrobenzoic acid) to give an initial absorbance of about 0.7 at 412 nm (50 mM sodium phosphate, 1 mM EDTA, pH 7.0, 22°). The reaction was initiated by the addition of alkylating agent.

Alkylating agent	*Initial concn. (mM)*	*Initial rate (ΔA/min)*
None	—	0
Bromopyruvate	0.1	0.153
N-Ethylmaleimide	0.1	0.126
Bromocitraconate	0.1	0.003
	1.0	0.028
Chlorolevulinate	0.1	0
	1.0	0.013
Iodoacetate	0.1	0
	1.0	0.012
Bromomesaconate	0.1	0
	1.0	0.006

2-nitro-4-thiolbenzoic acid at 22° and $1 \cdot 10^3$ to $2 \cdot 10^3$ $M^{-1} \cdot min^{-1}$ (apparent second order rate constant) for inactivation of succinate dehydrogenase.

Sensitive form of the enzyme

As prepared, soluble succinate dehydrogenase requires incubation with one of several compounds to achieve maximal activity[29]. Fig. 2 shows that the activated form of the enzyme is subject to attack by *N*-ethylmaleimide and bromopyruvate, whereas the nonactivated form (50 to 80% of the enzyme as prepared) is attacked only very slowly, if at all. The addition of these alkylating agents to the enzyme as prepared results in the prompt loss of most of the initial activity. If now an effective activating agent such as malonate is added, the net activity will increase. The magnitude of the increase depends on the relative rates of activation by malonate and irreversible inactivation by alkylating agent.

Inactivation by alkylating agents is retarded by malonate and other competitive inhibitors (see below). Note in Fig. 2 that the rate of inactivation upon the second

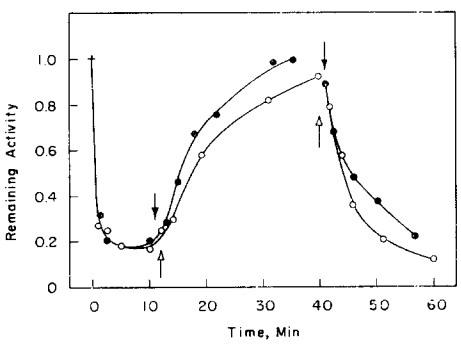


Fig. 2. The resistance of the unactivated form of succinate dehydrogenase to destruction by bromopyruvate and *N*-ethylmaleimide. The reaction mixtures contained 0.01 mM succinate dehydrogenase (not previously activated) and 1 mM alkylating agent added at time zero. Temperature, buffer, and pH were as in Fig. 1. ○, bromopyruvate; ●, *N*-ethylmaleimide. At the first open and first closed arrows, 1 mM malonate was added to activate the enzyme; at second open arrow, 5 mM additional bromopyruvate; at second closed arrow, 5 mM additional *N*-ethylmaleimide.

addition of bromopyruvate in the presence of malonate is less than the initial rate in the absence of malonate. Thus, in addition to activating succinate dehydrogenase, malonate also antagonizes the action of bromopyruvate as an irreversible inhibitor. The presence of residual malonate may explain why malonate-activated enzyme was inactivated more slowly than phosphate-activated enzyme (compare Figs. 1 and 3). When bromopyruvate and malonate were added together, the apparent activity of the enzyme increased or decreased initially, depending on the relative concentrations of these two compounds.

Reaction characteristics

As illustrated in Fig. 1, the inactivation of succinate dehydrogenase by bromopyruvate is characterized by an intitial rate which is concentration dependent and by a final residual activity or plateau which is also concentration dependent. The plateau

occurs also with *N*-ethylmaleimide. Both the initial rate of inactivation and the plateau level show a saturation effect such that neither is much affected by concentrations of bromopyruvate above about 0.5 mM. The saturation in rate suggests either the formation of a reversible complex between enzyme and bromopyruvate prior to inactivation or a change in rate determining step to one which is independent of the concentration of bromopyruvate. Since structurally dissimilar *N*-ethylmaleimide shows essentially the same rate and plateau concentration dependence (Fig. 3), the latter situation seems more likely.

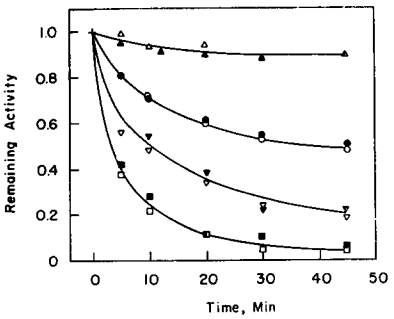


Fig. 3. The similarity of bromopyruvate and *N*-ethylmaleimide as inactivators of succinate dehydrogenase. The reaction mixtures contained 0.01 mM succinate dehydrogenase (previously activated by malonate) and alkylating agent as indicated. △, ○, ▽, □, bromopyruvate; ▲, ●, ▼, ■, *N*-ethylmaleimide. △, ▲, 0.2 mM; ○, ●, 0.5 mM; ▽, ▼, 1 mM; □, ■, 2 mM. Alkylating agents were added at time zero. Temperature, buffer, and pH were as in Fig. 1. The data for bromopyruvate have been corrected for a slight additional activation which occurred subsequent to the malonate treatment.

The most obvious explanation for a plateau in activity is that the concentration of bromopyruvate was drastically reduced early in the reaction, either through hydrolysis or by massive alkylation of protein. These possibilities were examined by several methods with essentially negative results: (a) Addition of bromopyruvate after the reaction appeared to level off caused little change in the residual activities (Fig. 1). (b) Reduction of the concentration of succinate dehydrogenase two fold to 5 μM did not influence the relative final activities. (c) Alkylation implies the linking of "pyruvate" to high molecular weight material. The concentration of free "pyruvate", measured as hydroxypyruvate[32] after the alkaline hydrolysis of bromopyruvate, was the same in incubation mixtures with and without succinate dehydrogenase. In addition, bromo[2-^{14}C]pyruvate was incorporated into protein only to the extent of a few moles/mole of enzyme. (d) The rapid reaction between bromopyruvate and 2-nitro-5-thiolbenzoic acid provided a means to estimate the concentration of surviving bromopyruvate directly. Except for an initial minor loss equivalent to 0.1 mM bromopyruvate or less, bromopyruvate survived in the presence of 10 μM succinate dehydrogenase under the usual experimental conditions for long periods of time and decayed with the half-life expected at pH 7. These experiments, taken together, indicate that bromopyruvate is not destroyed rapidly in the presence of succinate dehydrogenase preparations.

An alternative explanation for the kinetics of inactivation is that hydroxy-

pyruvate is generated and acts as an inhibitor of the alkylation reaction. No significant effect either on the shape of the inactivation curve at concentrations of 0.01–0.7 mM hydroxypyruvate or on the total amount of inactivation was observed.

Known competitive inhibitors of succinate dehydrogenase decrease both the initial rate and final extent of inactivation by bromopyruvate. The latter point is more clear from Table III in which results following 45 min incubations with bromopyruvate

TABLE III

THE PROTECTION OF SUCCINATE DEHYDROGENASE BY OXALOACETATE, MALONATE, AND SUCCINATE FROM INACTIVATION BY BROMOPYRUVATE

The reaction mixture (pH 7.0, 22°) contained one of the compounds specified, 0.01 mM succinate dehydrogenase, and 1 mM bromopyruvate. The enzyme used with oxaloacetate or without inhibitor was previously activated in phosphate buffer at 38°; that used with succinate or malonate was previously activated at 22° by the additive itself. Succinate, malonate, and oxaloacetate were added 1 h or more before bromopyruvate.

Concn. (M)	*Fraction of initial activity remaining after 45 min in the presence of bromopyruvate*			
	No addition	*+ Oxaloacetate*	*+ Malonate*	*+ Succinate*
0	0.10–0.13			
$1 \cdot 10^{-5}$		0.35		
$1 \cdot 10^{-4}$		0.62	0.23	
$1 \cdot 10^{-3}$		0.73	0.39	0.23
$1 \cdot 10^{-2}$			0.63	0.42
$1 \cdot 10^{-1}$				0.61

are tabulated. Protections by oxaloacetate, malonate, and succinate are concentration dependent, and the relative abilities of these compounds to block the action of bromopyruvate stand in the rank order of their relative binding strengths: succinate, $K_d = 0.065$ mM[23]; malonate, $K_d = 0.028$ mM[33]; and oxaloacetate, $K_d = 0.004$ mM[33]. The ability of these compounds to protect shows saturation characteristics as expected of compounds involved with bromopyruvate in competition for a specific site; however, it was not possible to demonstrate an approach to complete protection with either malonate or oxaloacetate.

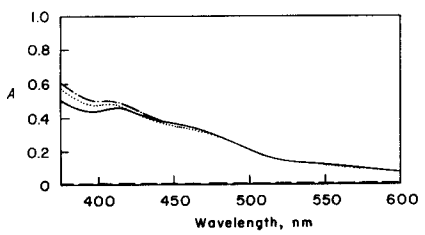


Fig. 4. Effect of bromopyruvate on the spectrum of succinate dehydrogenase. 0.01 mM succinate dehydrogenase, 150 mM phosphate buffer, pH 7.0, and 1 mM EDTA were contained in 1 ml cuvettes. ———, enzyme mixture before addition of bromopyruvate *vs.* water; · · · · ·, enzyme mixture *vs.* water 11 min after the addition of 1.7 mM bromopyruvate; –·–·—, same 28 min after addition of bromopyruvate. The spectra were recorded at room temperature with the cuvettes mounted in the scattering sample holder of a Unicam SP 800 spectrophotometer. The dashed line at $A = 0$ indicates enzyme mixture *vs.* itself before addition of bromopyruvate.

Changes in spectral and other properties following alkylation

Fig. 4 shows the change in the spectrum of a succinate dehydrogenase preparation during the course of the reaction with bromopyruvate. In contrast to the drastic changes that occur with mercurials[34], only minor changes were observed with an apparent isosbestic point at 416 nm. There was a small increase in absorbance below 416 nm and a much smaller decrease between 417 and 450 nm and between 500 and 600 nm. The eventual development of slight turbidity increased the absorbance at lower wavelengths and abolished the isosbestic point at 416 nm. The enzyme solution had lost 60% of its initial activity 10 min after the addition of bromopyruvate in the experiment illustrated in Fig. 4.

Also, as illustrated in Fig. 5, the effect of dithionite in reductively bleaching succinate dehydrogenase is only slightly modified after a prior incubation of enzyme with bromopyruvate.

Succinate also can bleach succinate dehydrogenase with a maximum effect at 465 nm, and the extent of bleaching under aerobic conditions with these preparations

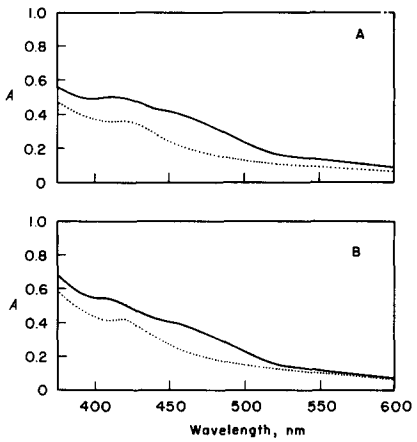


Fig. 5. The ability of dithionite to reductively bleach succinate dehydrogenase after inactivation by bromopyruvate. The reaction mixtures and instrument were as in Fig. 4. A, Without bromopyruvate: ———, enzyme mixture *vs.* water; · · · ·, enzyme mixture *plus* about 1 mM dithionite *vs.* water. B. With 1.7 mM bromopyruvate: ———, enzyme mixture *vs.* water 30 min after the addition of bromopyruvate; · · ·, same immediately after the addition of about 1 mM dithionite. Dithionite was added as the solid.

is about 15% of that accomplished by dithionite under anaerobic conditions. This ability of succinate to bleach succinate dehydrogenase is rapidly abolished by bromopyruvate, as illustrated in Fig. 6. The effect also appears to be irreversible.

The effect of alkylation on five other properties of succinate dehydrogenase were examined. The decrease in hydrogen exchange activity[35] using 1.4 mM 2,3-(*meso*)-[$^3H_2$]succinate as substrate paralleled the decrease in enzymatic activity of succinate dehydrogenase treated with bromopyruvate and dialyzed.

No qualitative difference in electrophoretic mobility in starch–agar between the treated and untreated enzyme was observed, only a decrease in the intensity of the activity-dependent stain.

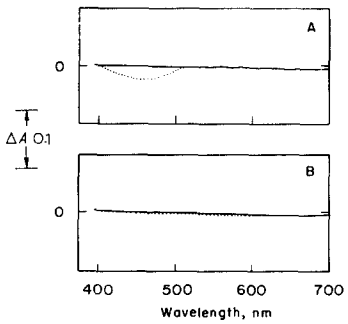


Fig. 6. The effect of bromopyruvate on the bleaching of succinate dehydrogenase by succinate. 0.008 mM succinate dehydrogenase, 150 mM phosphate buffer, pH 7.0, and 1 mM EDTA were contained in 1 ml cuvettes. Conditions were aerobic. Total initial volume was 1 ml. A. Without bromopyruvate: ———, enzyme mixture *vs.* itself; · · · ·, mixture *plus* 2.5 mM (10 μl) succinate *vs.* mixture *plus* 10 μl water. B. With 1 mM bromopyruvate; ———, enzyme mixture *vs.* itself 10 min after the addition of bromopyruvate to both cuvettes; · · · ·, same a few minutes after adding 2.5 mM (10 μl) succinate to sample cuvette and 10 μl water to reference cuvette. The spectra were recorded at room temperature with a Cary model 14 spectrophotometer.

The observed Michaelis constant at constant dye concentration appeared to vary with the per cent inactivation: 0.125 mM (0%), 0.057 mM (77%), and 0.037 mM (85%), indicating something other than an all-or-none effect of bromopyruvate.

While the iron atoms of succinate dehydrogenase are relatively inaccessible to iron chelators, reaction of the enzyme with *p*-chloromercuriphenylsulfonic acid allows at least 60% of them to be more rapidly removed by chelating agents[34]. To test the lability of iron after reaction with bromopyruvate, the enzyme was inactivated, precipitated with ammonium sulfate, washed four times, and subjected to iron analysis[36]. There was no difference in iron content between the treated succinate dehydrogenase and the untreated control sample (4 per peptide-bound flavin).

After incubating succinate dehydrogenase with bromo[2-^{14}C]pyruvate, the incorporated ^{14}C resists removal by gel filtration, trichloroacetic acid, and treatment with mercaptoethanol in denaturing agents. The label resists partial proteolysis and appears in low molecular weight compounds following acid hydrolysis. Covalent attachment seems likely.

DISCUSSION

Among the criteria for designation as an active-site-directed enzyme inhibitor are that: (1) the inactivation exhibits saturation with respect to inhibitor concentration; (2) the inactivation is impeded by substrates and competitive inhibitors; (3) high reactivity is expected relative to reaction with model compounds; and (4) a small and preferably stoichiometric number of covalent bonds are formed with residues on the enzyme. Bromopyruvate, the compound chosen for closest scrutiny as an inactivator, fulfills some, but not all, of these requirements.

While the saturation effect noted at high (1 mM) concentrations of bromopyruvate suggests the formation of an enzyme–modifier complex prior to reaction, the reaction kinetics were too complex to allow comparison of the data with predicted

behavior for this model[37]. Also, *N*-ethylmaleimide, which is very different in structure, shows almost identical saturation behavior.

The deviation from pseudo first order kinetics observed with bromopyruvate and the resulting activity plateau appear to be related to the nature of its reaction specifically with succinate dehydrogenase rather than a property of the reagent in general. Bromopyruvate has been reported as an active-site-directed inhibitor of *N*-acetylneuraminic acid aldolase[38] and 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase[39], exhibiting pseudo first order kinetics at pH 7.3 and 7.8, respectively. MELOCHE[16] reported deviations from first order kinetics at pH 6.0 in the inactivation of 2-keto-3-deoxy-6-phosphogluconate aldolase, but attributed this to enzymatic catalysis of the hydrolysis of bromopyruvate. HEINRIKSON *et al.*[40], explained deviations from expected second order kinetics in the reaction of bromopyruvate with histidine-119 of ribonuclease at pH 5.5 in terms of hydrolysis of the reagent. In the light of the apparent stability below pH 8 reported here, this interpretation might be questioned. Finally, bromopyruvate shows pseudo first order kinetics toward 2-nitro-5-thiolbenzoic acid dianion.

While succinate, malonate, and oxaloacetate protected the enzyme against inactivation by bromopyruvate, these compounds were never able to compete successfully enough to afford an approach to total protection, even at concentrations 100 times higher than their K_d values.

Succinate dehydrogenase appears to behave like the model sulfhydryl compound, 2-nitro-5-thiolbenzoic acid, with respect to the rate of attack by bromopyruvate, *N*-ethylmaleimide, and the other alkylating reagents. The rate of alkylation by bromopyruvate is not enhanced, as might be expected if it formed a complex with the enzyme prior to reaction.

The close similarities between bromopyruvate and *N*-ethylmaleimide in reaction kinetics, saturation effects, relative reactivities toward the enzyme and model compound, and degree of protection by reversible inhibitors are striking. In view of the strict steric requirements of this enzyme[7], the nearly identical behavior of two such structurally different molecules would seem to point to something other than an active-site-directed mechanism. In addition, the unactivated enzyme, which is alleged to have a kinetically retarded, but nevertheless competent active site[23], is not incapacitated by these two alkylating agents; activation and subsequent irreversible inhibition are still possible. Clearly the nucleophile and active site could be distant from each other in such a model.

It is likely that bromopyruvate does not extensively disrupt iron–sulfur bonds, judging by the relatively minor spectral changes accompanying alkylation compared to those reported by MASSEY[34], using *p*-chloromercuriphenylsulfonic acid. Reductive bleaching by dithionite still occurs after alkylation, although the detail of the spectrum is changed slightly. The main chromophores appear to remain largely intact.

The ability of bromopyruvate to eliminate bleaching by succinate and to inhibit hydrogen exchange is consistent with the idea that succinate no longer can react reductively with the enzyme and that this is a sufficient reason for loss of activity.

We recognize that active-site-directed inactivations can be complex and involve alkylation of any of a number of susceptible groups in the proximity of the site[41]. Taken as a whole, however, our data do not permit us to rank bromopyruvate and *N*-ethylmaleimide as active-site-directed inhibitors toward succinate dehydrogenase.

The data suggest in fact that inactivation may occur as the result of attack on groups located elsewhere and that certain events affecting the active site can also affect the reactivity or accessibility of crucial nucleophilic group(s).

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