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The effects of anions on fumarate reductase isolated from the cytoplasmic membrane of *Escherichia coli*

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A broad range of anions was shown to stimulate the maximal velocity of purified fumarate reductase isolated from the cytoplasmic membrane of *Escherichia coli*, while leaving the K_m for fumarate unaffected. Reducing agents potentiate the effects of anions on the activity, but have no effect by themselves. Thermal stability, conformation as monitored by circular dichroism and susceptibility to the thiol reagent 5,5′-dithiobis-(2-nitrobenzoic acid) are also altered by anions. The apparent K_m for succinate in the reverse reaction (succinate dehydrogenase activity) varies as a function of anion concentration, but the maximal velocity is not affected. The membrane-bound activity is not stimulated by anions and its properties closely resemble those of the purified enzyme in the presence of anions. Thus it appears that anions alter the physical and chemical properties of fumarate reductase, so that it more closely resembles the membrane-bound form.

Fumarate reductase acts as the terminal electron-transfer enzyme when *Escherichia coli* is grown anaerobically in the presence of glycerol and fumarate (Haddock & Jones, 1977). This membrane-bound enzyme has been solubilized and purified to 95% homogeneity in our laboratory (Dickie & Weiner, 1979; Lohmeier *et al.*, 1981). In addition to its fumarate reductase activity, the enzyme also catalyses a weak succinate dehydrogenase activity, the ratio of succinate oxidation to fumarate reduction being 0.03 (Dickie & Weiner, 1979).

We have found that a broad range of anions alter a number of physical and chemical properties of the purified enzyme, but not the membrane-bound form; the most conspicuous effect is the increase in the rate of fumarate reduction.

Materials and methods

Materials

5,5′-Dithiobis-(2-nitrobenzoic acid), ATP, ADP and AMP were from P-L Biochemicals, Milwaukee, WI, U.S.A. Sodium [^{3}H]cholate was from New England Nuclear, Boston, MA, U.S.A. *Escherichia coli* MV12/pLC 16-43, carrying a recombinant DNA plasmid coding for fumarate reductase, was grown anaerobically and fumarate reductase was purified from inner membranes as described previously (Lohmeier *et al.*, 1981).

Purified enzyme was depleted of cholate as follows: the enzyme solution was made 35% saturated with $(NH_4)_2SO_4$. After stirring for 1h at 0°C, the precipitated cholate was removed by centrifugation at 48000*g* for 20min. The enzyme was then precipitated by making the solution 60% saturated with $(NH_4)_2SO_4$ and stirring for 30min at 0°C. After centrifugation as described above, the dark amber pellet of fumarate reductase was dissolved in either 0.4M-potassium phosphate, pH6.8, or 25mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH6.8 (to give anion-depleted enzyme). This procedure removed up to 96% of the cholate present, as judged by the removal of [^{3}H]cholate.

Assay of enzyme activity

Fumarate reductase activity was assayed as described previously (Dickie & Weiner, 1979) except that the assay buffer was either 25mM-Hepes, pH6.8, or 200mM-sodium phosphate, pH6.8. Succinate dehydrogenase was assayed as described previously (Dickie & Weiner, 1979). Anions (titrated to pH6.8 with NaOH if necessary) were added directly to the assay buffer to the indicated final concentrations. Apparent K_m and $V_{max.}$ values were obtained from the Wilkinson (1961) statistical fit of initial-rate measurements.

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Treatment with 5,5'-dithiobis-(2-nitrobenzoic acid)

Fumarate reductase (300μg in 25mM-Hepes, pH6.8, or 270μg in 0.4M-potassium phosphate, pH6.8) was added to an equal volume of 40μM-5,5'-dithiobis-(2-nitrobenzoic acid) in 25mM-Hepes, pH6.8, and incubated at room temperature. Samples (2μl) were withdrawn at the indicated times and assayed for fumarate reductase activity.

Circular dichroism

Circular-dichroic spectra were measured between 200 and 250nm at 27°C in a Cary 60 spectrophotometer equipped with a 6001 CD attachment. Molar ellipticities were calculated as described by Oikawa *et al.* (1968) and the α-helical, β-sheet and random-coil components determined as described by Chen *et al.* (1974).

Other methods

Everted membrane vesicles were prepared as described by Rosen & Tsuchiya (1979) and resuspended in 38mM-Hepes, pH6.8.

Fluorescence measurements were performed with a Turner model 430 spectrofluorimeter. Flavin fluorescence was measured by exciting at 280nm and monitoring emission at 500nm.

Rate constants for the modification of fumarate reductase by 5,5'-dithiobis-(2-nitrobenzoic acid) were calculated from linear semi-log plots of activity versus time.

Results

Stimulation of fumarate reductase by anions

A broad range of uni- and bi-valent anions was found to stimulate the activity of purified fumarate reductase (Table 1). Although most anions tested stimulated activity, chaotropic anions such as perchlorate and thiocyanate inhibited the basal activity seen in the absence of activating anions. Phosphate, being one of the best activators, was selected for further detailed study (Fig. 1). In the absence of reducing agents 300mM-phosphate was required for maximal stimulation. However, addition of 0.5mM-dithiothreitol to the assay mixture caused both an increase in maximal activity and a lowering of the phosphate concentration required for maximal stimulation (to near 50mM). Other reducing agents, including β-mercaptoethanol and reduced glutathione, had a similar action. Interestingly, reducing agents had no effect in the absence of added anions, indicating that they apparently specifically potentiate the anion effect. Fig. 1 also shows that phosphate had no effect on the membrane-associated enzyme, regardless of whether reducing agents were present or not.

Fig. 2 shows the stimulatory effects of a series of phosphate esters. The order of potency was $PP_i > ATP > ADP > AMP$. All of the esters tested were better activators than P_i. We have also examined a series or organic acids with one, two or three carboxy groups. With this series the order of potency was citrate > malate > acetate (results not shown). It thus appears that the larger, more highly charged anions are superior activators. The stimulatory effect of anions occurred immediately (within 20s) when measured at 23°C and was reversible. The cholate form of the enzyme showed the same dependence of activity on anions as did the cholate-depleted form.

The *E. coli* fumarate reductase has a weak succinate dehydrogenase activity (Dickie & Weiner, 1979). The maximal activity of this reaction (20 units/mg) was not modulated by anions.

Table 1. *Stimulation of fumarate reductase by anions*

Enzyme (2.3μg), which had been dissolved in 25mM-Hepes, pH6.8, after cholate depletion, was used per assay. Stock salt solutions, at pH6.8, were added directly to the assay buffer to the indicated final concentrations. The assay buffer, 38mM-Hepes, pH6.8, contained 0.5mM-dithiothreitol.

Activator	Anion concn. ...	Stimulation (fold) 50mM	200mM
Sodium phosphate		5.20	5.86
NaN_3		5.20	5.80
Sodium citrate		4.2	5.80
Sodium acetate		3.44	5.23
Na_2SO_4		4.16	4.73
NaCl		2.25	3.74
KNO_3		ND	3.52
Sodium formate		2.58	3.50
$NaNO_3$		2.90	2.40
$NaSO_3$		1	1.32
Sodium borate		1	1.0

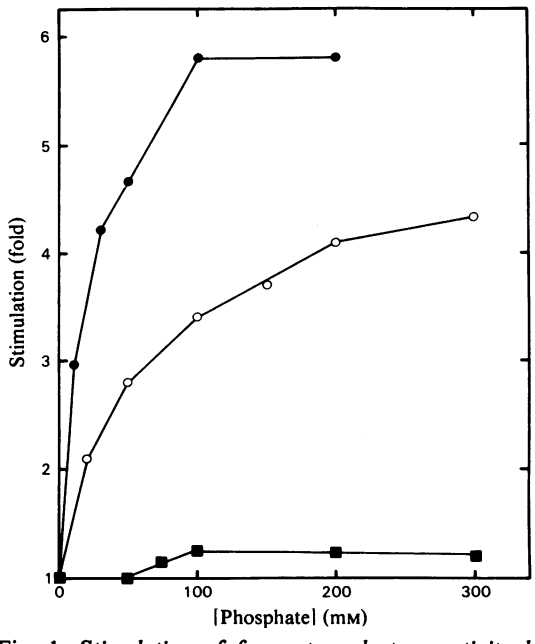

Fig. 1. *Stimulation of fumarate reductase activity by phosphate and dithiothreitol*

Assays were carried out as described in the Materials and methods section in 25mM-Hepes buffer, pH 6.8. Potassium phosphate, pH 6.8, was added to the indicated concentration. ■, Activity of vesicles (prepared with French press) in the presence or absence of 0.5mM-dithiothreitol. ○, Activity of purified enzyme in the absence of 0.5mM-dithiothreitol. ●, Activity of purified enzyme in the presence of 0.5mM-dithiothreitol. Activity is expressed as the stimulation (fold) above a basal value. For the membrane-bound and the purified enzyme 42 and 182 units/mg, respectively.

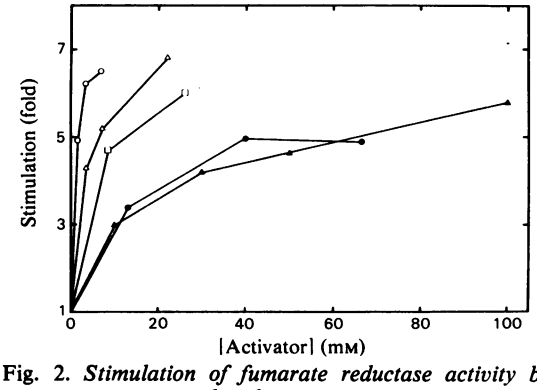

Fig. 2. *Stimulation of fumarate reductase activity by phosphate esters*

Assays were carried out as described above with purified enzyme. Activity in the presence of: ○, sodium pyrophosphate; △, ATP; □, ADP; ●, AMP; ▲, potassium phosphate. Activity is expressed as the stimulation (fold) above a basal value of 182units/mg.

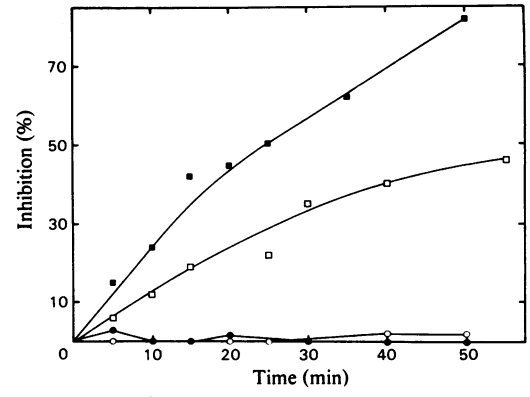

Fig. 3. *Thermostability of fumarate reductase*

Purified enzyme (2.1mg/ml) was incubated at either 40°C (□, ○) or 45°C (■, ●) in 25mM-Hepes, pH 6.8, in the absence (■, □) or presence (●, ○) of 200mM-potassium phosphate, pH 6.8. Samples were removed at the times indicated and assayed at 23°C as described in the Materials and methods section, except that the assay buffer contained 200mM-potassium phosphate, pH 6.8.

Effect of anions on thermostability

In the presence of phosphate, the enzyme was unaffected by incubation at 40° or 45°C for at least 1h (Fig. 3). However, in the absence of the anions, rapid loss of activity was observed. The thermal stability induced by anions closely paralleled the observed stability of the membrane-bound activity (results not shown).

Effect of anions on fumarate reductase fluorescence

The addition of potassium phosphate causes a quenching of nearly 30% of the flavin fluorescence. The titration of flavin fluorescence paralleled the stimulation of fumarate reductase activity, with maximal quenching at 200mM-potassium phosphate.

Effect of anions on the thiol sensitivity of fumarate reductase

Fumarate reductase contains a single thiol group, which is essential for catalytic activity (J. J. Robinson & J. H. Weiner, unpublished work). The second-order rate constant for modification with 5,5′-dithiobis-(2-nitrobenzoic acid) decreased from $56.5\,M^{-1}\cdot s^{-1}$ in the absence of phosphate to $20\,M^{-1}\cdot s^{-1}$ in the presence of phosphate. Release of the chromophore was followed at 412nm and closely paralleled the loss of activity. Control experiments showed that high ionic strength did not alter the rate of reaction of 5,5′-dithiobis-(2-nitrobenzoic acid).

Effect of anions on the circular-dichroic spectrum of fumarate reductase

The α-helix, β-sheet and random-coil components

of the enzyme in 25mM-Hepes, pH 6.8, were 34.8%, 17.4% and 47.8% respectively. In 0.4M-sodium phosphate, pH 6.8, the amounts were 30.1%, 12.9% and 57.0% respectively, indicating that the enzyme had a less-compact conformation in the presence of anions.

Effect on anions on the kinetic parameters of fumarate reductase

We measured the apparent K_m for fumarate as a function of phosphate concentration, and as Fig. 4 shows, the apparent K_m for fumarate (420μM) was unaltered by anion addition. This K_m closely resembles the dissociation constant, K_d, for fumarate binding to the enzyme, which was measured by following the quenching of flavin fluorescence as a function of fumarate concentration. A K_d of 450μM was observed in both the presence and absence of anions.

We measured the apparent K_m for succinate when solubilized fumarate reductase catalysed succinate dehydrogenase activity. Surprisingly, the apparent K_m for succinate rose substantially as anion was added to the assay buffer (Fig. 4). This increase in K_m brought about by phosphate followed a pattern similar to the stimulation of reductase maximal velocity. Similarly, when the membrane-bound fumarate reductase catalysed succinate oxidation, the K_m for succinate rose as a function of phosphate concentration, increasing from 53μM in 50mM-potassium phosphate to 1760μM in 200mM-potassium phosphate. Unfortunately, it was not possible to measure the K_d for succinate binding to the purified enzyme by fluorescence changes, as it failed to quench the endogenous flavin or tryptophan fluorescence.

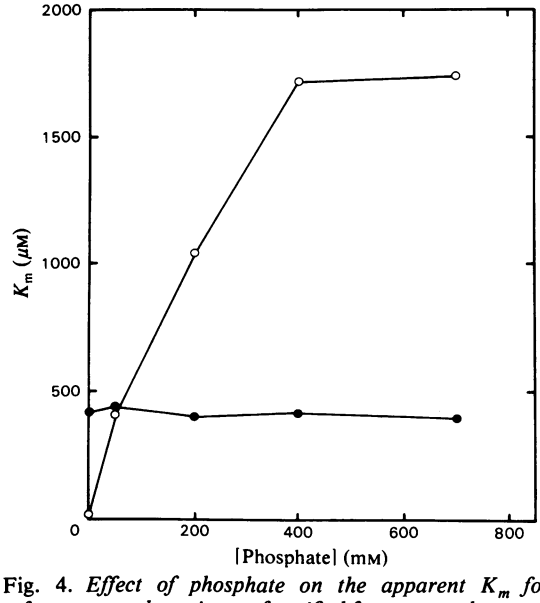

Fig. 4. *Effect of phosphate on the apparent K_m for fumarate and succinate of purified fumarate reductase*
The apparent K_m for succinate in the succinate dehydrogenase assay (○) or for fumarate in the fumarate reductase assay (●) was determined in an assay buffer containing various amounts of potassium phosphate. At least ten concentrations of succinate or fumarate were used for each K_m determination. The K_m was calculated by using the Wilkinson (1961) statistical fit of initial-rate data.

Discussion

In this paper we report several physical and catalytic properties of purified fumarate reductase which are modulated by anions. Addition of anions, particularly phosphate, to the enzyme preparation results in a dramatic increase in thermal stability, quenching of the endogenous flavin fluorescence, changes in the c.d. spectrum and a decrease in the susceptibility to the thiol reagent 5,5′-dithiobis-(2-nitrobenzoic acid). The last two results indicate a conformational change is occurring on activation, and the c.d. data suggest that the activated enzyme has a less-compact conformation. The maximal velocity of fumarate reduction is markedly enhanced by anions, whereas that of succinate oxidation is not affected. Interestingly, the properties reported for the solubilized enzyme in the presence of anions closely paralleled the properties of the membrane-bound enzyme where these could be measured. This led us to propose that anions were altering the structure of the enzyme so that the solubilized preparation more closely resembled the membrane-bound state.

Fumarate reductase is quite distinct from a large number of other membrane-bound enzymes (McIntyre *et al.*, 1978; Robinson & Weiner, 1980; Stephens & Gennis, 1980) in that the purified enzyme is not activated by non-ionic detergents. It is therefore possible that anions mimic the effect of phospholipid head groups in the membrane. For although it is difficult initially to release the enzyme from the membrane (Dickie & Weiner, 1979), the purified preparation binds the non-ionic detergent Triton X-100 poorly (J. J. Robinson & J. H. Weiner, unpublished work). This may indicate that a large part of the protein is not buried in the hydrophobic core, but rather is available at the surface to interact with phospholipid head groups. It is thus interesting that we have found that *O*-phosphonoethanolamine, the anionic head group of the major phospholipid class in *E. coli*, is an effective activator (5.2-fold activation at 19mM).

During the present work we observed that when the enzyme catalysed succinate oxidation the apparent K_m for succinate was markedly increased by

anions. This was true for both the highly purified enzyme and membrane-bound activity. Although this effect is probably distinct from the other alterations induced by anions, the overall result of the changes in apparent K_m and $V_{max.}$ reported here would be to shift the equilibrium in the direction of succinate formation in the presence of anions.

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