AN ESSENTIAL SULFHYDRYL GROUP AT THE SUBSTRATE SITE OF THE FUMARATE REDUCTASE OF *VIBRIO SUCCINOGENES*

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1. Introduction

Vibrio succinogenes contains a membrane-bound fumarate reductase which catalyzes the terminal step of the phosphorylative electron transport of the anaerobic bacterium. This enzyme has been isolated and characterized [1]. Here the response of the enzyme to sulfhydryl reagents is reported. As the electron transport from formate to fumarate is inhibited by a sulfhydryl reagent (*p*-CMS) [2], this is of interest with respect to the composition and organization of the electron transport chain and, therefore, relevant to the question of the mechanism of energy transfer [3].

2. Methods

The fumarate reductase containing cytochrome *b* which was prepared as in [1] was passed through a Sephadex G-25 column in order to remove DTT and fumarate. The column was equilibrated with an anaerobic solution containing 0.05% Triton X-100 and 20 mM Tris (pH 7.5 at 0°C).

The enzymic activities [1,3] and the contents of FAD [2], iron and sulfur [1] of fumarate reductase were measured as described.

Protein was measured by counting the radioactivity incorporated by growing the bacteria in the presence of [^{3}H]leucine. The specific activity (200–300 dpm/μg) of the protein was determined using the biuret method with KCN [1].

Abbreviations: *p*-CMS, 4-chloromercuriphenylsulfonate; NEM, *N*-ethyl-maleinimide; DTNB, 5,5'-dithio-bis(2-nitrobenzoate); DTT, dithiothreitol

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3. Results

The activity of reduction of methylene blue by succinate of a preparation of fumarate reductase containing cytochrome *b* [1] was measured after the enzyme had been incubated with increasing amounts of *p*-CMS (fig.1). The addition of 0.7 μmol *p*-CMS/g protein was without effect. On further addition, the activity decreased as a linear function of the amount of *p*-CMS added. The extrapolated line cut the abscissa at 5.7 μmol *p*-CMS/g protein. Thus the titre of *p*-CMS inhibition was determined to be 5.0 μmol/g protein. This number is considered to correspond to the content of a functional group of the enzyme which reacts with *p*-CMS. The contents of FAD and iron–sulfur were 6.0 and 41 μmol/g protein, respectively. The same titre of *p*-CMS inhibition was measured also with other activities of succinate oxidation and fumarate reduction [1] (not shown). In contrast, the activities of fumarate reduction by viologen dyes were not inhibited by *p*-CMS.

Succinate–methylene blue reduction was also inhibited by DTNB or NEM (table 1). As compared with *p*-CMS, however, greater amounts and longer incubation times were required in order to achieve $\geq$90% inhibition. The inhibition brought about by *p*-CMS or DTNB was reversed by the addition of DTT (table 1), dithionite or borohydride (not shown) while that caused by NEM was not. In contrast to *p*-CMS and DTNB, NEM brought about the inhibition of fumarate reduction by benzyl viologen radical (not shown).

When the enzyme was reacted with *p*-CMS or DTNB together with NEM, reactivation of enzyme activity with DTT was dependent on the order of the addition of the inhibitors (table 1). If *p*-CMS or DTNB were first applied to the enzyme, followed by

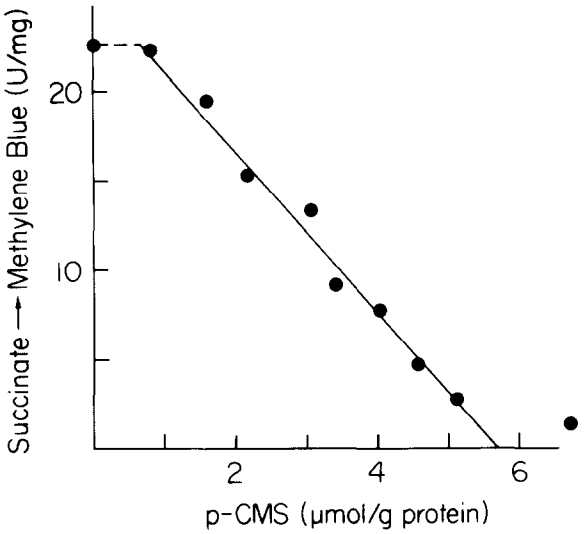

Fig.1. Titration with *p*-CMS of the activity of methylene blue reduction by succinate of fumarate reductase. The enzyme was incubated with *p*-CMS for 2 min in 10 mM Tris (pH 7.9 at 25°C) before the reaction was started by the addition of methylene blue and succinate.

the addition of NEM, 79% and 64% of the original activity was restored by DTT. In contrast, negligible degrees of reactivation were measured on the addition of DTT, if NEM was added before *p*-CMS or DTNB. This suggests that the reaction of NEM with the enzyme is prevented by *p*-CMS or DTNB and vice versa. Therefore, the inhibitors appear to attack the same site of the enzyme.

Table 1
Effect of sulfhydryl reagents on the activity of succinate–methylene blue reduction of fumarate reductase

Addition		Activity (%)	
First	Second	–	+ DTT
p-CMS		7	98
DTNB		9	86
NEM		8	10
p-CMS	NEM	6	79
DTNB	NEM	7	64
NEM	*p*-CMS	6	19
NEM	DTNB	4	13

The enzyme in a solution containing 0.05% Triton X-100 and 20 mM Tris (pH 7.9 at 0°C) was incubated with either *p*-CMS (14 μmol/g protein) for 5 min, DTNB (22 μmol/g protein) or NEM (40 μmol/g protein) for 20 min. When two reagents were applied, the excess of the first was removed by gel filtration, before the second was added. DTT was applied at 2 mM in the solution above. An activity of 100% corresponding to 16.5 μmol succinate . min^{-1} . mg $protein^{-1}$ was measured with or without treatment with DTT

Table 2
Determination of the content of sulfhydryl groups and cyanide binding of fumarate reductase

Incubation with	Amount bound (mol/mol FAD)	Succinate–methylene blue (U/mg)
–	–	20.3
DTNB	1.15	2.6
KCN (after DTNB)	1.04	2.4
KCN (1 mM)	0.10	18.2

The content of sulfhydryl groups was determined using DTNB [4]. Cyanide binding was measured using [^{14}C]cyanide. The enzyme was incubated for 15 min with 50 μmol DTNB/g protein in an anaerobic buffer containing 0.05% Triton X-100, 20 mM Tris (pH 7.9 at 0°C). After removing the excess DTNB by gel filtration using Sephadex G-25, the enzyme was incubated for 20 min with 1 mM Na[^{14}C]CN (10 750 dpm/nmol) in the above buffer, at 20°C. The excess cyanide was removed by gel filtration and the resulting enzyme preparation was analyzed for ^{14}C and ^{3}H (protein) using a double labeling program. The FAD content was 6.0 μmol/g protein

About 1 mol thionitrobenzoate dianion/mol FAD was released on reaction of fumarate reductase with DTNB and, concomitantly, the activity was 92% inhibited (table 2). This indicates that the inhibition is caused by reaction of a sulfhydryl group of the enzyme [4]. The enzyme inhibited by DTNB was reacted with labeled cyanide and the excess of cyanide was subsequently removed by chromatography on Sephadex G-25. Using the specific radioactivity of the cyanide, it was calculated that the enzyme contained ~1 mol cyanide/mol FAD. This indicates that the thionitrobenzoate residue which was bound to the enzyme has been exchanged for cyanide, thus confirming that the enzyme carries a reactive sulfhydryl group [5,6]. The cyanide containing fumarate reductase did not show enzymic activity. Incubation of the untreated fumarate reductase with cyanide did not result in the binding of a significant amount of cyanide and the enzymic activity was nearly unaffected.

The enzyme labeled with radioactive cyanide was subjected to gel electrophoresis in the presence of dodecylsulfate (fig.2) in order to determine the subunit to which the cyanide was bound. The gels were sliced and the slices were analyzed for cyanide label and protein. The cyanide was found to be associated with the M_r 79 000 peptide of the enzyme (0.32 mol

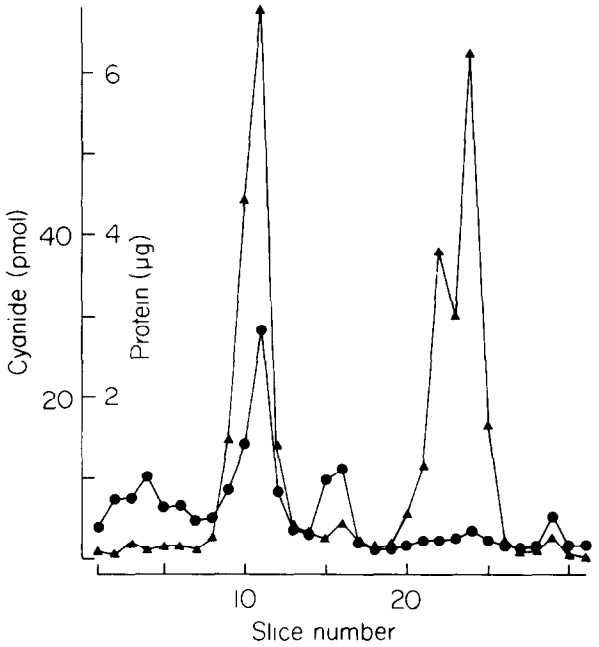

Fig.2. Gel electrophoresis in the presence of dodecylsulfate of fumarate reductase reacted first with DTNB and subsequently with [^{14}C]cyanide. The enzyme was incubated for 20 min with 25 μmol DTNB/g protein in an anaerobic solution containing 0.1% Triton X-100 and 20 mM Tris (pH 7.5 at 0°C). At this stage the enzyme activity was 6% of the original rate. Subsequently the solution was brought to 20°C, adjusted to pH 9 by the addition of Tris and 1.5 mM Na[^{14}C]CN (10 750 dpm/nmol) was added. After 25 min the solution was passed through a Sephadex G-25 column in order to remove the excess of cyanide. At this stage the enzyme contained 2.65 μmol cyanide/g protein. A sample (44 μg protein) was incubated for 1 h in a solution containing 10% dodecylsulfate, 40 mM borate and 40 mM Tris (pH 8.5 at 20°C) then subjected to gel electrophoresis according to [1,7]. The gel was sliced and the slices digested with 'NCS-solubilizier' (Amersham/Searle, Arlington Height, IL). The digest was analyzed for ^{3}H (protein) (▲) and ^{14}C (●) by scintillation counting using a double labeling program.

cyanide/mol), while the M_r 31 000 and the M_r 25 000 subunits were free of cyanide. On reaction of fumarate reductase with labeled NEM and gel electrophoresis as before, the inhibitor was again present only in the larger subunit which carries the FAD (not shown).

The incubation period with the inhibitiors was increased by the presence of the substrates of the enzyme. This is demonstrated with DTNB in fig.3. In the absence of substrate, 50% inhibition was measured after incubation for 3 min. In order to obtain the same degree of inhibition in the presence of either 40 mM succinate or 3.5 mM fumarate, incubation for 8 and 18 min was required, respectively. ≳90% inhibition was measured under all the conditions after incubation for >40 min. The inhibition curve with 0.5 mM fumarate present (not shown) was nearly equal to that measured in the presence of 40 mM succinate. Thus the concentrations of the substrates that exert the same effect show about the same relation as the Michaelis constants (0.35 mM fumarate and 20 mM succinate [1]). Malonate as a competitive inhibitor of the enzyme also delayed the inhibition, while malate, maleinate, acetate or sulfate were without effect (not shown). These results indicate that the site of inhibition is close to the substrate site of the enzyme.

4. Discussion

These results show that the fumarate reductase of *V. succinogenes* contains a sulfhydryl group at an amount equivalent to that of FAD. The sulfhydryl group is localized on the M_r 79 000 subunit of the enzyme that carries the FAD. The reaction of this group with sulfhydryl reagents is specifically inhibited by the substrates of the enzyme fumarate and succinate as well as by the competitive inhibitor malo-

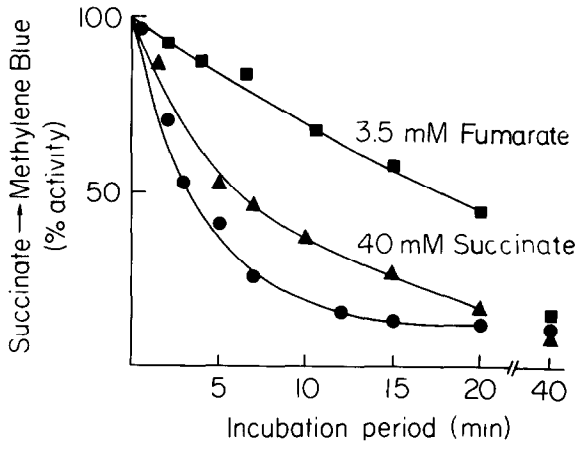

Fig.3. Time course of DTNB inhibition of succinate–methylene blue reduction in the absence and presence of the substrates of fumarate reductase. The enzyme was incubated in an anaerobic solution containing 0.05% Triton X-100 and 20 mM Tris (pH 7.5 at 0°C) with or without (●) the substrates present and 12.5 μmol DTNB/g protein were added. After the time periods indicated samples were removed and assayed for enzymic activity. An activity of 100% corresponded to 17.6 μmol succinate . min^{-1} . mg protein^{-1}.

nate. This suggests that the sulfhydryl group is localized at the substrate site of the enzyme. Furthermore, this confirms the earlier conclusion that fumarate and succinate interact at the same site [1].

The group appears to be essential for enzymic activity, since >90% inhibition was achieved with each of 3 different sulfhydryl reagents, and replacement of the bulky thionitrobenzoate residue by the much smaller cyanide group did not lead to the restoration of enzymic activity. The latter result appears to exclude the possibility that the inhibition is a consequence merely of steric hindrance of the substrates from entering the active site [6].

The inhibition of fumarate reductase by *p*-CMS, which does not penetrate through the membrane, can be used for elucidating the orientation of the substrate site of the membrane-bound enzyme. As reported [3], the enzyme of inverted vesicles from *V. succinogenes* was >90% inhibited by *p*-CMS, while that of cells and spheroplasts was not affected at all. This indicates that the sulfhydryl group and consequently the substrate site faces the cytoplasm of the bacterium, in agreement with the conclusion drawn in [3]. In [2] the titre of *p*-CMS inhibition was overestimated, because under the conditions used the reaction of fumarate reductase with *p*-CMS was not complete. From the titre thus obtained and from the observation that fumarate reduction by viologen radicals was insensitive to *p*-CMS, it was erroneously concluded that the iron–sulfur groups of fumarate reductase were attacked by the inhibitor [2]. This misinterpretation is corrected here on the basis that fumarate reduction by viologen radicals is inhibited by NEM and that NEM attacks the same site as *p*-CMS. The finding that fumarate reduction by viologen radicals was not inhibited by *p*-CMS may be explained by a reversal of the binding of the inhibitor to the enzyme which is caused by the viologen radicals. It is likely that viologen radicals exert a similar effect on the bound inhibitors to DTT, dithionite or borohydride.

The catalytic properties of the enzyme of *V. succinogenes* are clearly those of a fumarate reductase. Nevertheless it is structurally similar to the mitochondrial succinate dehydrogenase [1,8]. This pertains also to the presence of the sulfhydryl group at the substrate site. However, while fumarate reductase contains only 1 reactive sulfhydryl group, at least 1 additional reactive sulfhydryl group apart from the substrate site has been detected in succinate dehydrogenase [9].

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