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New properties of *Bacillus subtilis* succinate dehydrogenase altered at the active site

The apparent active site thiol of succinate oxidoreductases is dispensable for succinate oxidation

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Mammalian and *Escherichia coli* succinate dehydrogenase (SDH) and *E. coli* fumarate reductase apparently contain an essential cysteine residue at the active site, as shown by substrate-protectable inactivation with thiol-specific reagents. *Bacillus subtilis* SDH was found to be resistant to this type of reagent and contains an alanine residue at the amino acid position equivalent to the only invariant cysteine in the flavoprotein subunit of *E. coli* succinate oxidoreductases. Substitution of this alanine, at position 252 in the flavoprotein subunit of *B. subtilis* SDH, by cysteine resulted in an enzyme sensitive to thiol-specific reagents and protectable by substrate. Other biochemical properties of the redesigned SDH were similar to those of the wild-type enzyme. It is concluded that the invariant cysteine in the flavoprotein of *E. coli* succinate oxidoreductases corresponds to the active site thiol. However, this cysteine is most likely not essential for succinate oxidation and seemingly lacks an assignable specific function. An invariant arginine in juxtaposition to Ala-252 in the flavoprotein of *B. subtilis* SDH, and to the invariant cysteine in the *E. coli* homologous enzymes, is probably essential for substrate binding.

INTRODUCTION

Mammalian succinate dehydrogenase (SDH) (EC 1.3.99.1) is known since 1938 (Hopkins & Morgan, 1938; Hopkins *et al.*, 1938), or perhaps earlier (Thunberg, 1916), as being very sensitive to reagents that modify thiol groups (see Vinogradov, 1986; Hatefi, 1985; Singer *et al.*, 1973, for reviews). Inactivation of SDH activity by such reagents results from the modification of one unusually reactive Cys residue of unknown position in the largest protein subunit of the enzyme (Kenney, 1975; Vinogradov *et al.*, 1976). Substrate and substrate analogues, such as malonate, confer protection against the inactivation. These findings suggest that an essential Cys residue in SDH is located at or very close to the active site. The thiol of this Cys has been proposed to be directly involved, as a proton donor/acceptor, in the catalytic mechanism of SDH (Vinogradov, 1986; Vinogradov *et al.*, 1976). By comparative studies and chemical modification experiments combined with site-directed mutagenesis of *Bacillus subtilis* SDH we demonstrate in this work that the reactive Cys is not essential for succinate oxidation.

SDH is a membrane bound iron-sulphur flavoenzyme of central importance in aerobic cells. It catalyses the oxidation of succinate to fumarate in the tricarboxylic acid cycle, with direct transfer of reducing equivalents to the respiratory chain. Anaerobic organisms, which can use fumarate as ultimate electron acceptor, often contain a membrane-bound fumarate reductase (FRD) (EC 1.3.99.1) that *in vivo* catalyses the opposite enzymic reaction, i.e. reduction of fumarate to succinate. Facultative bacteria such as *Escherichia coli* can synthesize both a SDH and a FRD (Miles & Guest, 1987).

The composition and primary structure of succinate oxidoreductases (SDH and FRD enzymes) from different organisms are similar (Hatefi, 1985; Ohnishi, 1987; Phillips *et al.*, 1987). They are composed of a catalytic part consisting of one 60–79 kDa flavoprotein (Fp or A) subunit and one 25–31 kDa iron–sulphur protein (Ip or B) subunit. One FAD is covalently bound to the Fp subunit, whereas the Ip subunit seems to harbour three different iron–sulphur centres (Ohnishi, 1987; Johnson *et al.*, 1987). One or two transmembrane proteins (a cytochrome *b* or C and D polypeptides) anchor Fp and Ip to the membrane and are essential for quinone reduction.

The reactive thiol is located on the Fp subunit as shown by radioactive labelling studies on isolated bovine heart SDH (Kenney *et al.*, 1976), *E. coli* FRD (Robinson & Weiner, 1982; Ackrell *et al.*, 1987) and *Wolinella succinogenes* FRD (Unden & Kröger, 1980). Furthermore, Robinson & Weiner (1982) have, for *E. coli* FRD, mapped this thiol to the middle part of the Fp polypeptide. *E. coli* SDH also contains a substrate protectable thiol (the present paper) but the subunit location of the reactive group has in this case not been demonstrated. Only one invariant Cys is present in the primary structure of Fp from *E. coli* FRD and SDH (Fig. 1). This residue, located adjacent to an Arg in a conserved sequence about 250 amino acid residues from the *N*-terminus, has been proposed as the reactive thiol which, when chemically modified, inactivates the enzyme (Wood *et al.*, 1984; Cole *et al.*, 1985).

The nucleotide sequence of the *sdhCAB* operon encoding cytochrome b_{558}, Fp and Ip in the Gram-positive bacterium *B. subtilis* has been determined (Magnusson *et al.*, 1986; Phillips *et al.*, 1987). The derived amino acid sequences have been confirmed by *N*-terminal sequence

Abbreviations used: SDH, succinate dehydrogenase; FRD, fumarate reductase; Fp, flavoprotein; Ip, iron–sulphur protein; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); NEM, *N*-ethylmaleinimide.

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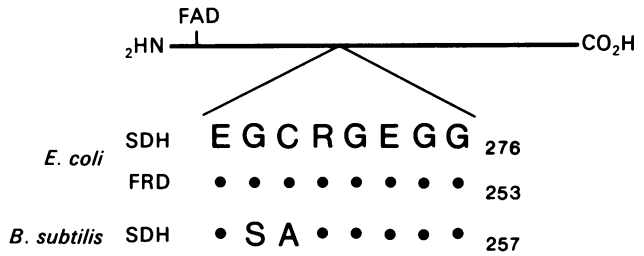

Fig. 1. Schematic illustration of the Fp subunit of succinate oxidoreductases and amino acid sequence comparison of one invariant region

The Cys in this sequence in *E. coli* SDH and FRD has tentatively been identified as the active site thiol. Amino acids are in one-letter code. A dot indicates the same amino acid as in *E. coli* SDH. Data are from: Cole (1982), Wood *et al.* (1984) and Phillips *et al.* (1987).

analysis (Hederstedt *et al.*, 1987). Fp of *B. subtilis* SDH and of *E. coli* SDH and FRD contain identical residues at 32% and 35% of the positions, respectively. However, the Cys residue tentatively identified as being the active site thiol in *E. coli* succinate oxidoreductases is replaced by Ala in *B. subtilis* SDH (Fig. 1). This unexpected finding initiated this work where the identity and role of the long-studied thiol is examined by a molecular genetic approach.

MATERIAL AND METHODS

Bacteria and growth of bacteria

B. subtilis and *E. coli* strains used are listed in Table 1. *B. subtilis* strains were kept on Tryptose Blood Agar Base (Difco) plates. Acid accumulation by *B. subtilis* strains was analysed by streaking cells on Purification Agar plates (Carls & Hanson, 1971). Liquid cultures of *B. subtilis* strains were grown at 37 °C in Nutrient Sporulation Medium Phosphate as described before (Hederstedt, 1986), except that the pH was 7.0 and $MnCl_2$ was not added to the medium. *E. coli* carrying plasmid was grown in LB broth (Miller, 1972) containing ampicillin (35 μg/ml) or kanamycin (50 μg/ml). *E. coli* AN345 was grown at 37 °C aerobically in Davis minimal salt medium supplemented with 1% (w/v) sodium succinate, pH 7.4, 0.4 mM-$MgCl_2$, thiamin (1 μg/ml), proline and leucine (20 μg/ml).

Preparation of membranes

B. subtilis membranes were isolated from osmotically lysed cells (Hederstedt, 1986). The washed membranes were suspended in 20 mM-Mops buffer, pH 7.4, or 50 mM-Hepes buffer, pH 7.4, and stored at −80 °C. *E. coli* membranes were isolated from spheroplasts prepared from exponentially growing cells essentially as described by Kaback (1971). The spheroplasts were isolated by centrifugation at 7000 *g* for 10 min at 20 °C and lysed in 1 mM-sodium EDTA/50 mM-potassium phosphate buffer, pH 6.6, by sonication on an ice-bath. The lysate was then diluted 10-fold in 5 mM-$MgSO_4$/50 mM-potassium phosphate, pH 6.6, containing 2.5 μg of DNAase/ml and incubated at 30 °C for 30 min. Cell debris was removed by centrifugation at 5000 *g* at 4 °C for 15 min. Membranes in the supernatant were collected by centrifugation at 50000 *g* at 4 °C for 30 min, and washed once in 20 mM-Mops buffer, pH 7.4, before they were frozen in liquid N_2.

Enzyme activity measurements

SDH activity was determined at 30 °C as the succinate-dependent reduction of phenazine methosulphate (Hatefi, 1978). The cuvette routinely contained 50 mM-potassium phosphate or 50 mM-Tris/chloride buffer, pH 7.4, and 20 mM-potassium succinate, pH 7.4, 1 mM-KCN, 0.1 mM-sodium EDTA, 0.5 mg of phenazine methosulphate/ml and 0.02 mg 2,6-dichlorophenolindophenol/ml as terminal electron acceptor (ϵ 21 $mM^{-1} \cdot cm^{-1}$ at 600 nm). The enzyme reaction was started by the addition of 5–20 μl of membranes to 1 ml final volume. For determinations of turnover number, enzyme activity was measured at different concentrations (0.05–0.5 mg/ml) of phenazine methosulphate and the $V_{max.}$ activity related to the histidyl flavin content of the preparation (Singer, 1971).

B. subtilis membrane preparations containing de-

Table 1. Bacterial strains and plasmids

		Relevant genotype	Source or reference
B. subtilis	SDHΔ12	Δ(*sdhCA*)	Fridén *et al.* (1987*a*)
	LU2600	*sdhC*$^+$ *sdhA*$^+$ *sdhB*$^+$	The present work
	LU2617	*sdhC*$^+$ *sdhA17 sdhB*$^+$	The present work
	LU2618	*sdhC*$^+$ *sdhA18 sdhB*$^+$	The present work
	LU2619	*sdhC*$^+$ *sdhA19 sdhB*$^+$	The present work
	168W	Prototroph	Laboratory stock
E. coli	AN345	*pro leu*	MacGregor (1976)
	JM83	Δ(*lac–proAB*) *lacZ* M15	Yanisch-Perron *et al.* (1985)
	JM103	Δ(*lac–proAB*)/F′, *traD36 proAB lacI*^{q}*Z* M15	Yanisch-Perron *et al.* (1985)
	ES871	*strA lacZ* (ICR 36) *trpA540 mutL*/F′*lac pro*	P. Carter
Plasmids	pUC18	*bla*	Yanisch-Perron *et al.* (1985)
	pUC19	*bla*	Yanisch-Perron *et al.* (1985)
	pSH1047	*Km*^{r} *Cm*^{r} *sdhC*$^+$ *sdhA*$^+$ *sdhB*$^+$ *gerE*$^+$	Hasnain *et al.* (1985)
	pBSD2600	*bla sdhC*$^+$ *sdhA*$^+$ *sdhB*$^+$ *gerE*$^+$	The present work
	pBSD2617	*bla sdhC*$^+$ *sdhA17 sdhB*$^+$ *gerE*$^+$	The present work
	pBSD2618	*bla sdhC*$^+$ *sdhA18 sdhB*$^+$ *gerE*$^+$	The present work
	pBSD2619	*bla sdhC*$^+$ *sdhA19 sdhB*$^+$ *gerE*$^+$	The present work

activated SDH due to tightly-bound oxaloacetate were activated by incubation in 0.4 M-NaBr/10 mM-Hepes/40 mM-potassium phosphate, pH 6.6, at 25 °C (Kearney *et al.*, 1974) and then resuspended in 50 mM-Hepes, pH 7.4, after centrifugation at 48000 *g* at 4 °C for 40 min. *E. coli* membrane-bound SDH was activated similarly, but in 0.1 M-potassium phosphate, pH 7.4, at 38 °C. No further increase in SDH activity during incubation in the presence of 20 mM-succinate at 30 °C was used as the criterion for full activation.

Chemical modification

Membranes, about 1.5 mg of protein/ml in 50 mM-Hepes, pH 7.4 (if no other buffer is stated), were pre-incubated at 30 °C. A small volume (less than 7 % of the final volume) of stock solution of modifying reagent or only solvent was added at time zero. Samples were withdrawn at time intervals and immediately analysed for enzyme activity. The dilution of the sample in the spectrophotometer cuvette stopped further inactivation caused by the modifying reagent. Stock solutions of 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) and diacetyl were prepared in absolute ethanol, dansyl chloride in acetone and *N*-ethylmaleinimide (NEM) in Hepes buffer. The concentration of NEM was determined using the absorbance coefficient 620 $M^{-1} \cdot cm^{-1}$ at 305 nm (Riordan & Vallee, 1967). All reagents were obtained from Sigma Chemical Co.

DNA techniques and transformation of bacteria

Plasmids used are listed in Table 1. pUC18, pUC19 and derivatives thereof were propagated in *E. coli* JM83. Plasmid DNA and the replicative form of phage M13 DNA were isolated by the procedure of Ish-Horowicz & Burke (1981). Digestion by endonuclease restriction enzymes, ligation by T4 ligase and agarose gel electrophoresis of DNA were done according to standard methods (Maniatis *et al.*, 1982). Competent *E. coli* (Hanahan, 1983) and *B. subtilis* (Arwert & Venema, 1973) were prepared as described before.

In vitro mutagenesis

The method for site-specific mutagenesis on M13 was essentially as described (Carter *et al.*, 1985). The template was obtained by first cloning a 1.3 kbp *Pst*I–*Hin*dIII fragment from pSH1047 into pUC18 in *E. coli* JM83. The resulting plasmid was digested with *Hin*dIII followed by a partial *Eco*RI digestion and the 495 bp internal *sdhA* fragment was isolated from low gelling temperature (LGT) agarose (Crouse *et al.*, 1983) and inserted into M13 mp18 (Messing, 1983). Single-stranded DNA from phage propagated on *E. coli* JM103 was prepared as described (Messing, 1983) and used in primer extension with the synthetic oligonucleotides shown in Fig. 3. Plaques obtained after transformation of *E. coli* ES871 were then screened for hybridization to the mutagenesis primers end-labelled with ^{32}P (Maniatis *et al.*, 1982). Phages giving hybridization were picked and the nucleotide sequence of the insert determined. The replicative form was prepared from clones containing the correct sequence and a 290 bp *Sst*II–*Nco*I fragment was isolated from LGT agarose and used to replace the corresponding wild-type sequence in pBSD2600.

Analytical methods

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard. DNA (Maniatis *et al.*, 1982), covalently bound flavin (Hederstedt, 1980) and Fp antigen (Rutberg *et al.*, 1978) were determined as described elsewhere. DNA sequences were analysed using the dideoxy chain termination method (Sanger *et al.*, 1977).

RESULTS

Sensitivity of *B. subtilis* and *E. coli* SDH to chemical modification

To compare biochemical properties of SDH containing and lacking the tentatively identified active site Cys (Fig. 1) we analysed *E. coli* and *B. subtilis* SDH for sensitivity to NEM and DTNB. The results are shown in Table 2. *E. coli* SDH showed the same property as FRD (Ackrell *et al.*, 1987; Robinson & Weiner, 1982) and mammalian SDH (Kenney, 1975; Vinogradov, 1986) in that it was inactivated by both reagents and was protected by substrate and substrate analogues (Fig. 2). In contrast, *B. subtilis* SDH was not sensitive to either of the reagents. The active site thiol of mammalian SDH is sensitive at pH 6 to dansyl chloride (Hederstedt & Hatefi, 1986), a reagent which generally is not specific for Cys. Membrane-bound *E. coli* SDH was likewise much more sensitive than *B. subtilis* SDH to dansyl chloride at

Table 2. Sensitivity of *E. coli* and *B. subtilis* wild type SDH to reagents modifying thiol and guanido groups

Membranes were incubated with the given reagent in 50 mM-potassium phosphate, pH 7.4, for 15 min at 30 °C. The SDH activity was then assayed in phosphate buffer. The control is membranes incubated under the same conditions but without modifying reagent. The specific SDH activity of the 100 % controls for *E. coli* and *B. subtilis* were 1.28 and 1.36 μmol of succinate oxidized/min per mg of protein, respectively.

Reagent	Concentration (mM)	SDH activity remaining (% of control)		Reactive residue
		E. coli AN345	*B. subtilis* 168W	
NEM	0.06	45	100	Cys
	0.6	10	100	
DTNB	0.01	28	100	Cys
	1.3	3	100	
Diacetyl	45	27	12	Arg
	90	14	2	

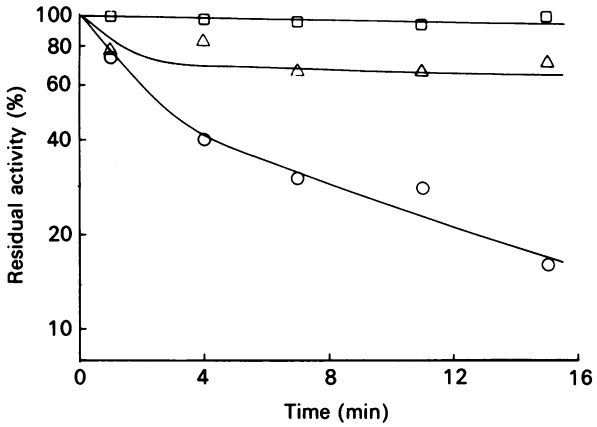

Fig. 2. Time course of inactivation of *E. coli* AN345 membrane-bound SDH by NEM

Membranes in 50 mM-potassium phosphate buffer, pH 7.4, were incubated with 0.5 mM-NEM in the absence (○) and in the presence of 13 mM-succinate (△) or 13 mM-malonate (□).

pH 6.0, and substrate protected against inactivation of the *E. coli* enzyme (results not shown). All these findings are as expected if the Cys-256 in Fp of *E. coli* SDH is the sensitive target for modification by thiol-specific reagents.

The resistance of *B. subtilis* membrane-bound SDH to NEM and DTNB was not due to a general inaccessibility of this enzyme to chemical modification. Incubation of membranes in the presence of diacetyl inactivated SDH activity (Table 2). Diacetyl has been shown to modify essential substrate-protectable Arg in mammalian SDH (Kotlyar & Vinogradov, 1984). Both *B. subtilis* and *E. coli* SDH were similarly protected by succinate and malonate against inactivation by this reagent (see below).

Site-directed mutagenesis of *B. subtilis* SDH

For a better understanding of the functional role of amino acid residues in the homologous sequence of Fp shown in Fig. 1 we redesigned *B. subtilis* SDH to become more similar to the *E. coli* succinate oxidoreductases. Ser-251 and Ala-252 in the Fp subunit were replaced by Gly and Cys, respectively, individually and both at the same time, i.e. three new enzyme variants were constructed. *B. subtilis* SDH now containing Cys-252 was predicted to show the following properties if the corresponding Cys in *E. coli* SDH and FRD had been correctly identified as very reactive to NEM and DTNB and located at the active site: (i) to be enzymically active, (ii) to be sensitive to low concentrations of thiol-modifying reagent, and (iii) to be protectable by substrate and substrate analogues against the modification.

The desired amino acid substitutions in Fp were obtained by synthetic oligonucleotide mutagenesis in phage M13 as outlined in Fig. 3. The nucleotide changes were confirmed by DNA sequence analysis of the phage M13 clones. The wild type *B. subtilis sdh* operon, on a *Bam*H1–*Sal*I DNA fragment isolated from pSH1047, was cloned in pUC19 to yield pBSD2600. Unique *Sst*II and *Nco*I endonuclease restriction sites in *sdhA* were subsequently used to replace the wild type sequence in pBSD2600 by the DNA fragments isolated from the M13 clones (Fig. 3). The resulting plasmids, pBSD2617, pBSD2618 and pBSD2619, contained the *sdh* operon with the introduced mutations *sdhA17*, *sdhA18* and *sdhA19*, respectively. The plasmid constructs were confirmed by restriction enzyme mapping and by hybridization of plasmid DNA to the same oligonucleotides as used for the site-directed mutagenesis.

Phenotype of mutants

B. subtilis strain SDHΔ12, which has the *sdhC* and *sdhA* genes deleted, was transformed with pBSD2600 and its three derivatives. Transformation with these plasmids, which cannot replicate autonomously in *B. subtilis*, assured that the *sdhC* and *sdhA* genes of the plasmids were integrated into the chromosome in one copy by homologous recombination across *sdhB* or the flanking *gerE* DNA. Sdh$^+$ transformants were selected on agar plates containing citrate and glutamate as carbon

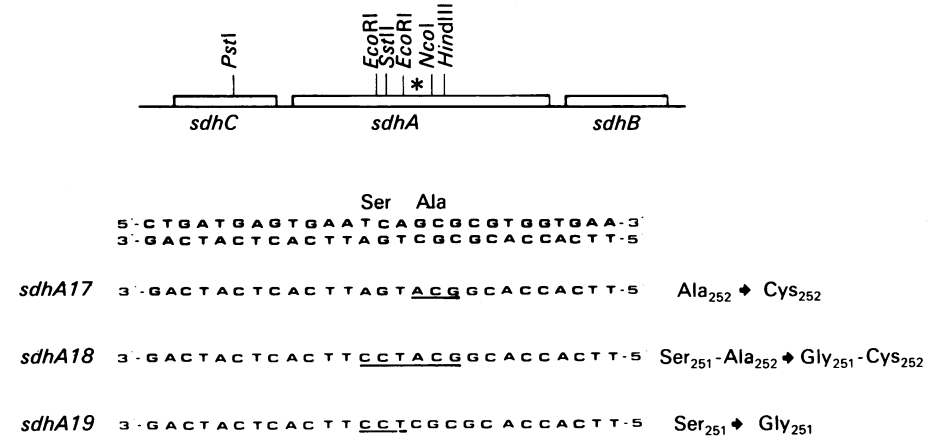

Fig. 3. Genetic map of the *B. subtilis sdh* operon and outline for site-directed mutagenesis

Only restriction enzyme sites employed in subcloning and mutagenesis are indicated. The star in *sdhA* indicates the location of the DNA sequence shown underneath which encodes the amino acids 247–252 in the Fp subunit. The three synthetic oligonucleotide primers used for mutagenesis and the resulting *sdhA* mutations and predicted amino acid substitutions are presented.

Table 3. Kinetic properties of wild type and redesigned membrane-bound *B. subtilis* SDH

The amino acid residues indicated for each enzyme variant are those at positions 251 and 252 in the Fp subunit. The *B. subtilis* strains from which the membranes were prepared are given within parentheses. Strain LU2600 contains wild-type SDH. Turnover numbers ($k_{cat.}$) were determined as mol of succinate oxidized/mol of covalently bound flavin per s for three independent membrane preparations from each strain. The apparent K_m and K_i values were calculated from Lineweaver–Burke plots. Enzyme activity was measured in 50 mM-potassium phosphate buffer, pH 7.4, at 30 °C as described in the Materials and methods section.

Property	Enzyme variant			
	-Ser-Ala- (LU2600)	-Ser-Cys- (LU2617)	-Gly-Cys- (LU2618)	-Gly-Ala- (LU2619)
$k_{cat.}$ (s^{-1})	90–98	53–55	42–58	18–27
$K_m^{app.}$ for succinate (mM)	0.9	0.5	0.5	0.9
$K_i^{app.}$ for malonate (mM)	0.10	0.08	0.08	0.03

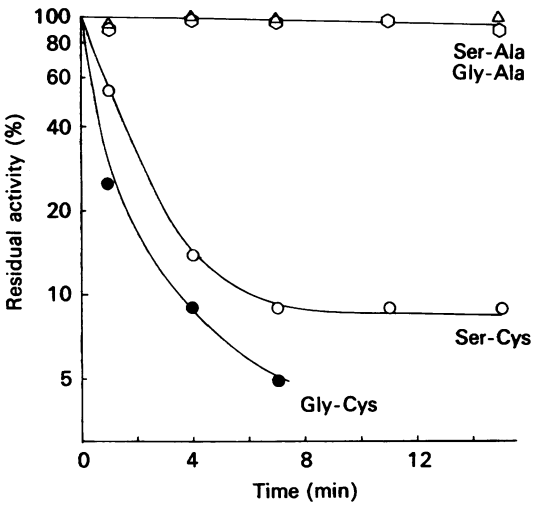

Fig. 4. Time course of inactivation of membrane-bound *B. subtilis* wild-type and redesigned SDH by NEM

The two indicated amino acids denote those at positions 251 and 252 in the Fp subunit of the respective SDH variant. LU2600 (wild type) was incubated with 980 μM-NEM (○); LU2617, 71 μM-NEM (○); LU2618, 71 μM-NEM (●); LU2619, 980 μM-NEM (△).

and energy source (Fridén *et al.*, 1987*b*). All four plasmids resulted in transformants and at similar frequencies, which demonstrated that the three mutant Fp polypeptides could be assembled into functional membrane-bound SDH. One Sdh^+ transformant obtained with the respective plasmid was kept (Table 1 and Fig. 3).

The constructed strains grew as well as the wild type equivalent, LU2600, on solid and in liquid media. They did not accumulate acid as SDH-defective *B. subtilis* do (Hederstedt, 1986). Membrane preparations of LU2618 and LU2619, however, consistently contained only 30–80% the amount of SDH protein compared with LU2600 or LU2617, as determined by immunoelectrophoresis against anti-Fp serum and by covalently bound flavin. SDH is the only protein with covalently bound flavin in *B. subtilis* membranes (Hederstedt, 1983). Reduced amounts of SDH protein have also been observed in membranes from other mutants with a single amino acid substitution in Fp (Maguire *et al.*, 1986).

Catalytic properties of mutant SDH

Kinetic properties of SDH in membranes from wild-type *B. subtilis* and from the three constructed mutants are summarized in Table 3. The turnover number for redesigned SDH was 20–50% lower than for the wild-type enzyme. Substitution of Ala-252 by Cys alone or together with Ser-251 by Gly did not affect the apparent second-order rate constant, $k_{cat.}/K_m$, of the enzyme. Exchange of Ser-251 for Gly, only, had a stronger effect than the double substitution and caused an about 4-fold decrease in apparent $k_{cat.}/K_m$.

Chemical modification of mutant SDH

To determine if Cys-252 in Fp affects the sensitivity of SDH to thiol modifying reagents, membranes of each *B. subtilis* mutant were treated with NEM under conditions which inactivated *E. coli* SDH. Both enzyme variants with Cys at this position were inactivated at low concentrations of NEM, whereas the control enzymes with Ala-252 were not affected even at a 10-fold higher NEM concentration (Fig. 4). Furthermore, succinate and malonate protected both sensitive enzyme variants against the effect of NEM (Fig. 5). The dicarboxylic acid, potassium 3,3-dimethylglutarate, which is not a substrate analogue for SDH, did not confer protection. This showed that the protection by succinate and malonate was specific and seemingly at the active site. Qualitatively the same results as with NEM were obtained when the mutant enzymes were treated with DTNB (10 μM range) or with dansyl chloride (100 μM range) in 0.1 M-phosphate buffer, pH 6.0 (results not shown).

Chemical modification experiments on mammalian SDH suggest that an essential Arg is located at the active site and close to the reactive thiol (Kotlyar & Vinogradov, 1984). This Arg may correspond to that in juxtaposition to the invariant Cys in Fp of *E. coli* SDH and FRD, and at position 253 in *B. subtilis* Fp (Fig. 1). Substitution of Ala-252 by Cys in *B. subtilis* Fp could therefore affect the reactivity of the guanido group of Arg-253 towards diacetyl and if so would provide additional evidence for the location and role of the essential Arg in SDH. The wild type and all three variants of *B. subtilis* SDH were found to be sensitive to diacetyl, and substrate protected against the inactivation (Table 4). The inhibition rates differed slightly in that SDH with Cys next to Arg-253 appeared more rapidly inactivated than enzyme with Ala adjacent to the Arg.

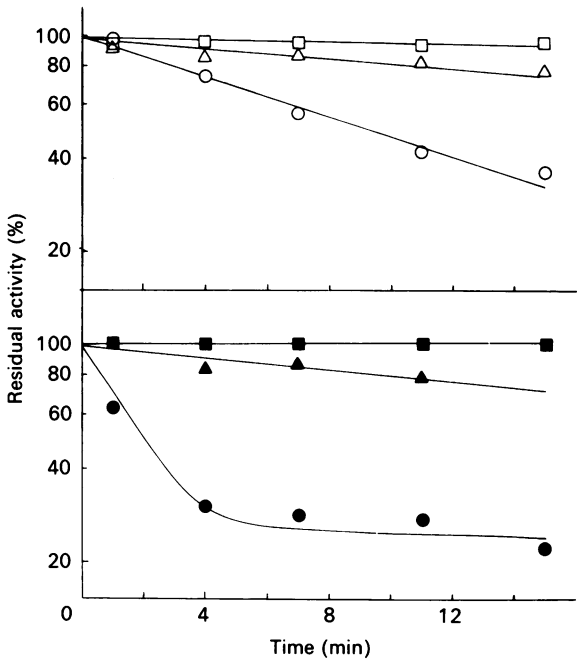

Fig. 5. Protection of redesigned *B. subtilis* SDH against inactivation by NEM

Membranes of LU2617 (Ser-Cys) (top panel) and LU2618 (Gly-Cys) (lower panel) were incubated with 14 μM-NEM in Hepes buffer (○, ●) and in the buffer supplemented with 18 mM-potassium succinate (△, ▲) or 12 mM-potassium malonate (□, ■).

DISCUSSION

Substitution of Ala-252 by Cys in the Fp subunit of *B. subtilis* SDH confered a new specific property to the enzyme: it became sensitive to low concentrations of thiol-modifying reagents. In addition, succinate and malonate protected the redesigned SDH against the inactivation. Other properties of the Ala→Cys altered enzymes, such as the specificity constant ($k_{cat.}/K_m$) and substrate-protectable sensitivity to Arg modifying reagent, remained essentially unchanged compared with wild-type SDH. These results strongly suggest, but do not prove, that residue 252 in the Fp subunit is located at or very close to the substrate binding site of *B. subtilis* SDH.

B. subtilis SDH with Cys-252 thus mimics the property of *E. coli* SDH and FRD, and also mammalian SDH, with respect to sensitivity to thiol modifying reagents. The primary structure of mammalian Fp is not known, but those of *E. coli* SDH and FRD have only one invariant Cys (Wood *et al.*, 1984). The location of this Cys corresponds to residue 252 in the *B. subtilis* Fp subunit (Phillips *et al.*, 1987). These data together leave little doubt that Cys-248 and Cys-271 in Fp of *E. coli* FRD and SDH, respectively, are identical with the active site thiol. *Proteus vulgaris* FRD has this Cys at position 247 (Cole, 1987) and mammalian SDH is predicted to contain a Cys at the corresponding position in Fp.

More important is the finding that this active site Cys is not required for succinate oxidation as evidenced by the catalytic properties of wild-type *B. subtilis* SDH. If not essential for structural or catalytic function, what is the role of the thiol when present? Mammalian SDH and *E. coli* succinate oxidoreductases can tightly bind oxaloacetate and are thereby reversibly inactivated (Ackrell *et al.*, 1974, 1987). The deactivation has been proposed to result from oxaloacetate forming a thiohemiacetal with the active site thiol (Vinogradov *et al.*, 1972). This role for the thiol seems incorrect, because *B. subtilis* SDH with Ala and Cys, respectively, at position 252 in Fp are both deactivated by oxaloacetate (L. Hederstedt & B. A. C. Ackrell, unpublished work). Apparently the Cys has no specific function, but its location on the enzyme is by coincidence such that substrate, directly or indirectly, prevents it from reacting with NEM and DTNB. The lack of enzyme activity of SDH modified at the thiol may result from direct steric hindrance of substrate binding by the thiol adduct or from blockage of essential conformational changes in the enzyme.

Supported by double chemical modification experiments on mammalian SDH, Kotlyar & Vinogradov (1984) have suggested that Arg functions in substrate binding and proposed that the high reactivity of the active site thiol originates from close location to an Arg side chain. As shown in this work, *B. subtilis* and *E. coli* SDH also contain substrate-protectable Arg. There are nine invariant Arg residues in the Fp polypeptide of *B. subtilis* SDH, *E. coli* SDH and FRD and *P. vulgaris* FRD (Cole, 1982, 1987; Wood *et al.*, 1984; Phillips *et al.*, 1987). The invariant Arg-253 in *B. subtilis* Fp is probably

Table 4. Inhibition of membrane-bound *B. subtilis* wild-type and redesigned SDH by diacetyl and protection by substrate

k, pseudo-first-order inhibition rate constant. The amino acid sequence indicated for each enzyme variant is that for residues 251, 252 and 253 in the Fp subunit. Membranes were incubated with 53 mM-diacetyl at 30 °C in the absence and presence of 20 mM potassium succinate and 20 mM potassium malonate. The diacetyl was added to the membranes from a 0.75 M stock solution in ethanol. Final concentration of ethanol was 7% (v/v). The inhibition rates are corrected for the small inhibition caused by ethanol only.

		k (min^{-1})		
Membrane	Enzyme variant	No protective agent	+Succinate	+Malonate
LU2600 (wild type)	-Ser-Ala-Arg-	0.38	0.15	< 0.04
LU2617	-Ser-Cys-Arg-	0.58	0.23	< 0.04
LU2618	-Gly-Cys-Arg-	0.46	0.15	< 0.04
LU2619	-Gly-Ala-Arg-	0.20	0.05	< 0.04

a substrate-protectable Arg. This conclusion is based on its location adjacent to the substrate-protectable Cys in the Gram-negative enzymes and on the fact that substitution of residue 251 and/or 252 in *B. subtilis* Fp affected the reactivity of SDH to modification by diacetyl. It should be noted that the Ser-251 → Gly substitution seemingly perturbs the active site, since not only was the rate of inhibition by diacetyl the lowest for this enzyme, but also the $k_{cat.}/K_m$ was low compared with wild-type and the other enzyme variants. In conclusion, Cys, Arg and His residues have been indicated as essential residues at the active site of SDH (Kenney, 1975; Vik & Hatefi, 1981; Kotlyar & Vinogradov, 1984; Hederstedt & Hatefi, 1986). We can now exclude Cys. The side chain of Arg-253 in *B. subtilis* Fp possibly has a role in substrate binding by forming an ionic pair with one of the substrate carboxyl groups. His-235 and/or His-381 in Fp may perform proton donor/acceptor functions in the catalytic mechanism (Phillips *et al.*, 1987). FAD is covalently bound to His-40 in the Fp subunit of *B. subtilis* SDH (Phillips *et al.*, 1987). The indicated active site amino acid residues are thus not in the primary structure located in the vicinity of His-40.

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