IMMUNOCHEMICAL ANALYSIS OF THE MEMBRANE-BOUND SUCCINATE DEHYDROGENASE OF *ESCHERICHIA COLI*

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1. Introduction

The membrane-bound succinate dehydrogenase (EC 1.3.99.1) of *Escherichia coli* has proved difficult to isolate due to its low degree of stability when solubilized from the membrane [1]. Crossed immunoelectrophoresis has proved a valuable technique in the characterization of membrane-bound enzymes which are not readily purified [2–5]. Here we describe the application of this technique to the study of succinate dehydrogenase from *E. coli* and show that the enzyme contains non-haem iron and consists of 2 subunits of M_r 73 000 and 26 000. A preliminary report of this work has been published [6].

2. Materials and methods

2.1. *Cell growth, membrane preparation and solubilization*

Escherichia coli strain MR43L/F152, which carries the F152 episome [7], was grown at 37°C with vigorous shaking in the basal medium of [8] supplemented with 1 mM $MgSO_4$, 0.15% (w/v) casamino acids and 0.5% (w/v) sodium succinate. The succinate dehydrogenase-deficient mutant CBT312 [9] which is available from the *E. coli* Genetic Stock Centre (Yale University, New Haven, CT 06510) was grown in the same medium except that sodium lactate replaced sodium succinate and the medium was additionally supplemented with thiamine (20 μg/ml). For growth on ^{55}Fe, iron was omitted from the basal medium and the medium was supplemented with 1.5 μM $^{55}FeCl_2$ at a final activity of 200 μCi/l. The growth medium for the ^{35}S-labelled culture consisted of 0.3 g Bacto-yeast extract (Difco Labs.), 1.36 g K H_2PO_4, 0.4 g NH_4Cl, one grain of $FeSO_4$, 1 ml 0.3 mM $MgSO_4$, 0.1 ml 0.1 mM $CaCl_2 \cdot H_2O$, 1 mCi $H_2{}^{35}SO_4$, 0.5 g sodium succinate in a final volume of 100 ml, at pH 7.0. All subsequent steps were done in the presence of 5 mM benzamidine and 10 mM phenylmethyl sulphonyl fluoride. Isolation of the cytoplasmic membrane was as in [10]. Membranes were solubilized in Triton X-100 and protein was measured as in [2].

2.2. *Immunization of rabbits and preparation of antibody*

The procedure for immunizing rabbits, collecting serum and isolating the antibody fraction was as in [2].

2.3. *Electrophoretic techniques*

The equipment, preparation of Triton X-100-solubilized extracts and procedures for both isoelectric focusing (IEF) and crossed immunoelectrophoresis have been described [2]. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was performed using 13% polyacrylamide by the Laemmli method [10]. 'Western blotting' [12] was carried out by a modification of the method in [13].

2.4. *Activity stains*

The succinate dehydrogenase stain employed was that in [14].

2.5. *Materials*

Phenazine methosulphate and tetranitroblue tetrazolium were purchased from Sigma. Agarose (Sea Kem™HGT) and Gel Bond Film were purchased from FMC Corp. (Rockland, ME 04841). Sources of other reagents and equipment have been described [2].

Abbreviation: IEF, isoelectric focusing

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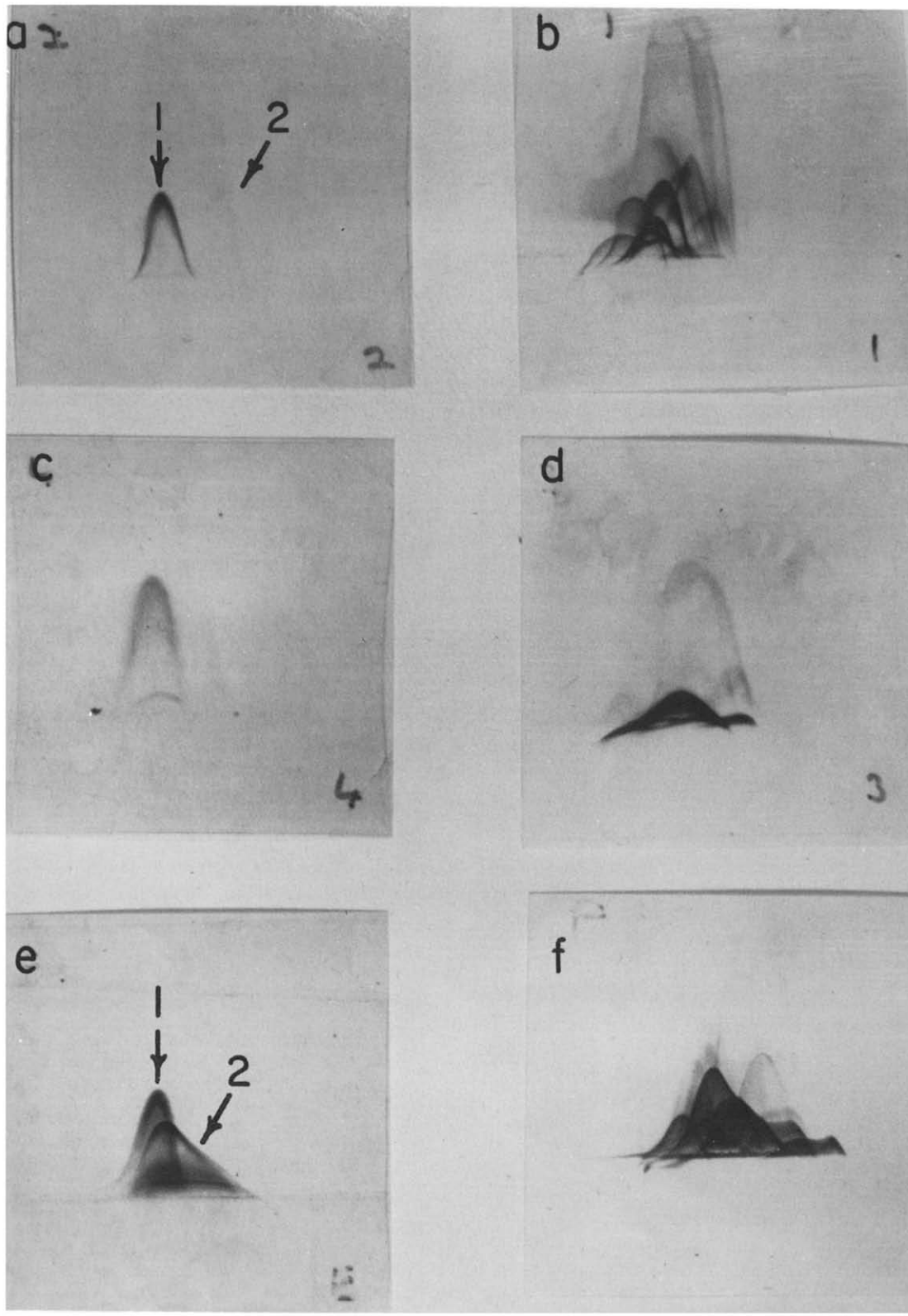

Fig.1.

3. Results

3.1. *Isoelectric focusing of succinate dehydrogenase*

Isoelectric focusing of a Triton X-100-solubilized cytoplasmic membrane extract gave two bands which stained for succinate dehydrogenase activity (not shown). One, the major band, had pI 5.7 and appeared in every gel that was run. The other minor band had pI 7.2 and was not always observed. The bands were excised and used to immunize separate rabbits.

3.2. *Crossed immunoelectrophoretic analysis of succinate dehydrogenase*

Analysis by crossed immunoelectrophoresis of a Triton X-100-solubilized membrane extract against antiserum raised to the major IEF band revealed several precipitin arcs which stained for protein (fig.1b) of which one (arc 1, fig.1a) stained heavily for succinate dehydrogenase activity. A second minor arc (arc 2, fig.1a) was also observed, but this took much longer to activity stain.

Analysis of a Triton X-100-solubilized extract against the antiserum raised to the minor IEF band showed a different protein pattern (fig.1d) but revealed only one succinate dehydrogenase-specific arc (fig.1c). This succinate dehydrogenase arc appeared at the same point as arc 1 observed with the serum raised to the major IEF band. Analysis of the Triton X-100-solubilized membrane extract against a mixture of the 2 antisera gave only one major succinate dehydrogenase-staining arc (not shown). Crossed immunoelectrophoresis was also run in which the second dimension consisted of a layer of agarose containing only one antiserum, followed by a layer containing only the other antiserum. The results (not shown) again indicated that the succinate dehydrogenase-specific antibodies in the 2 antisera are similar. The titre of succinate dehydrogenase-specific antibody was ~10-fold higher in the antiserum to the major IEF band than in that raised against the minor IEF band.

Analysis of the Triton X-100-solubilized extract against antiserum raised to the isolated inner membrane showed a more complex protein pattern (fig.1f). In this case, one of the 2 succinate dehydrogenase-stained precipitin arcs (arc 1, fig.1e) appears to correspond to the predominant arc (arc 1) of fig.1a. The other arc (arc 2, fig.1e) corresponds to arc 2 of fig.1a. This was confirmed by crossed immunoelectrophoretic analysis of a membrane extract against a mixture of the antiserum directed against the inner membrane and the antiserum against the major IEF band (not shown). Fig.1e clearly shows that arcs 1 and 2 do not contain any common antigen since the arcs are not fused but cross one another.

3.3. *Prosthetic groups*

An ^{55}Fe-labelled Triton X-100-solubilized membrane extract was analyzed against the antiserum to the major IEF band. Autoradiography revealed 5 iron-containing arcs, of which one corresponded to the succinate dehydrogenase-staining arc (fig.2). As a control, the ^{55}Fe-labelled extract was analyzed by crossed

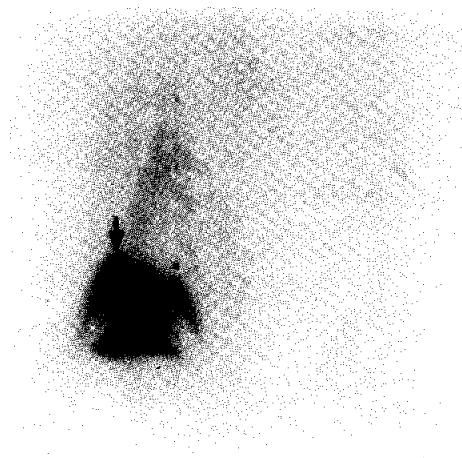

Fig.2. ^{55}Fe-Containing antigens: An ^{55}Fe-labelled membrane extract (70 μg protein) was analyzed by crossed immunoelectrophoresis against 50 μl of the antiserum raised to the major IEF band. The plate was then autoradiographed. The succinate dehydrogenase-specific arc is arrowed.

Fig.1. Crossed immunoelectrophoretic analysis of succinate dehydrogenase. A Triton X-100-solubilized membrane extract (a,b,e,f = 90 μg protein; c,d = 18 μg) was analyzed by crossed immunoelectrophoresis against 75 μl antiserum raised to the major IEF band (a,b), 300 μl antiserum raised to the minor IEF band (c,d) or 250 μl antiserum raised against isolated inner membrane (e,f). Plates were stained for succinate dehydrogenase activity (a,c,e) or protein (b,d,f). The succinate dehydrogenase-staining arcs are arrowed (see text).

immunoelectrophoresis against antibody specific for lactate dehydrogenase, an enzyme which has been shown not to contain iron [15]. A lactate dehydrogenase-staining arc was obtained, but no arc was detected by autoradiography. This observation indicates that the iron detected in the arcs of fig.2 is not attributable merely to non-specific binding but actually represents iron incorporated into the enzyme as a prosthetic group. Activity-staining [2] failed to detect any haem-containing arcs precipitated by this

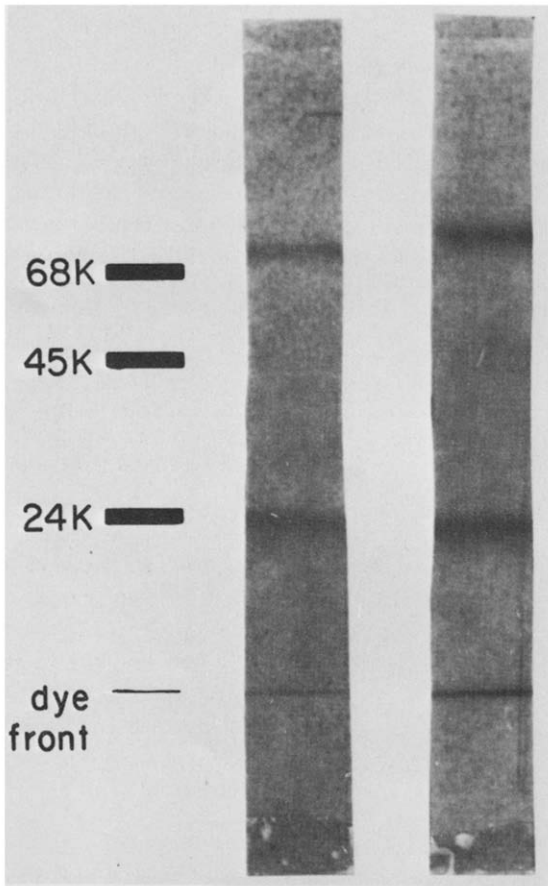

Fig.3. Subunit composition of succinate dehydrogenase. An ^{35}S-labelled membrane extract was analyzed by crossed immunoelectrophoresis against antiserum to either the major IEF band or to isolated inner membrane. Segments of the succinate dehydrogenase-specific arc which were free from other protein were excised and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography. The left lane shows the positions of M_r standards bovine serum albumin, ovalbumin and chymotrypsinogen. The centre lane is from the arc precipitated by the antiserum to the major IEF band and the right lane from the corresponding arc precipitated by the antiserum to the isolated inner membrane.

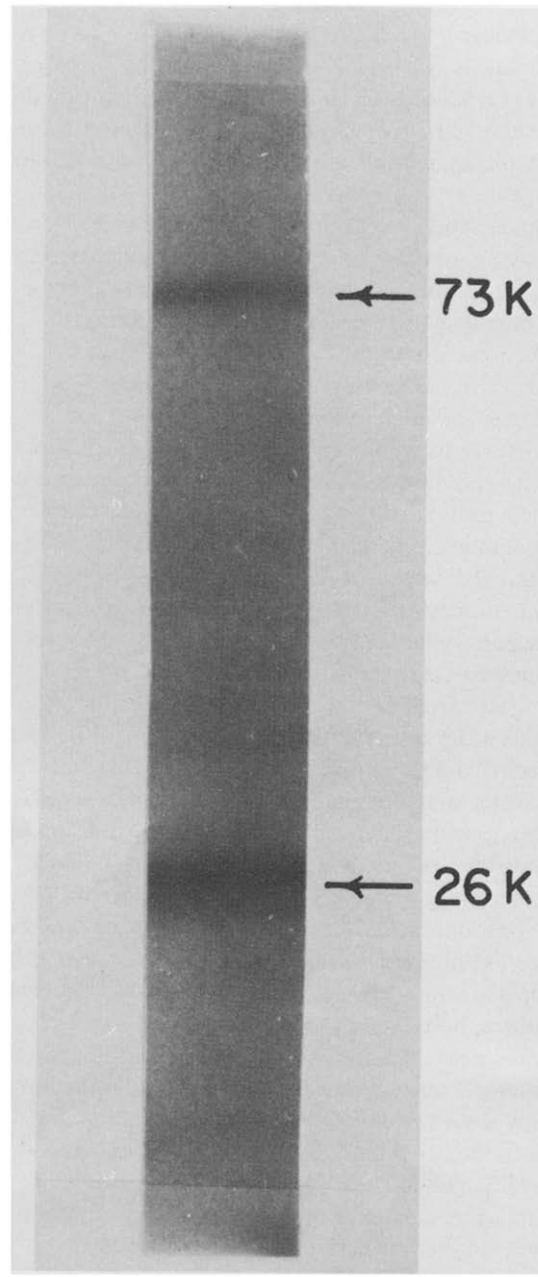

Fig.4. Analysis of succinate dehydrogenase by 'Western blotting'. A Triton X-100-solubilized membrane extract was analyzed by crossed immunoelectrophoresis against the antiserum to the major IEF band. Segments of the succinate dehydrogenase-specific precipitin arc which were free of other contaminating proteins were excised and analyzed by SDS–polyacrylamide gel electrophoresis. Proteins were then electrophoretically transferred to nitrocellulose. The nitrocellulose was incubated with the antiserum to the major IEF band and then labelled with ^{125}I-protein A. The nitrocellulose was then autoradiographed. M_r standards were those used in fig.3.

antiserum. Analysis of a membrane extract from a 5-aminolaevulinic acid-requiring mutant grown in the presence of 5-[^{3}H]-aminolaevulinic acid [2] also failed to reveal any haem-containing arcs precipitated by the antiserum raised to the major IEF band.

3.4. *Subunit composition of succinate dehydrogenase*

A ^{35}S-labelled Triton X-100-solubilized extract was analyzed by crossed immunoelectrophoresis against each of the 3 antisera. The portions of the succinate dehydrogenase-staining arc 1 which were free of additional protein-staining material were excised and analyzed by SDS–polyacrylamide gel electrophoresis, followed by autoradiography. The autoradiograph (fig.3, center lane) showed that the ^{35}S-labelled succinate dehydrogenase-staining material precipitated by the antiserum directed against the major IEF band consisted of 2 subunits of M_r 73 000 and 26 000. Autoradiography of the lane containing the corresponding arc (arc 1) precipitated by antiserum to the inner membrane showed the same 2 ^{35}S-labelled subunits (fig.3, right lane).

Polypeptides were also analyzed using a procedure recently referred to as 'Western blotting' [12]. Proteins separated by SDS–polyacrylamide gel as in fig.3 were electrophoretically transferred to nitrocellulose. After incubation with the antiserum directed against the major IEF band and subsequent labelling with ^{125}I-protein A, the nitrocellulose was autoradiographed. Fig.4 shows that the succinate dehydrogenase-specific antibody in this antiserum contains antibody to 2 polypeptides of M_r 73 000 and 26 000.

3.5. *Analysis of a succinate dehydrogenase-negative mutant*

Strain CBT312 [9] had <5% of the succinate dehydrogenase activity of wild-type strain MR43L/F152. If this strain were to contain an inactive succinate dehydrogenase antigen, the precipitin arc could not be readily identified by activity staining. To overcome this problem we used the technique of tandem crossed immunoelectrophoresis. Two wells were cut side-by-side and one well was loaded with a Triton X-100 extract of membranes from the wild-type strain. In the other well was loaded an equal amount of Triton extract of the mutant strain. After crossed immunoelectrophoresis, fused arcs are obtained. The succinate dehydrogenase-specific precipitin arc from the wild-type strain is then activity stained. Fig.5 shows that the arc which is fused with the succinate dehydrogenase-staining arc from the wild-type strain is very small. Since the area under the arc is proportional to the amount of antigen present, it can be concluded that the mutant CBT312 contains <10% of the succinate dehydrogenase-specific antigen found in the wild-type strain MR43L/F152. Succinate dehydrogenase levels in MR43L/F152 were the same whether grown on succinate or lactate.

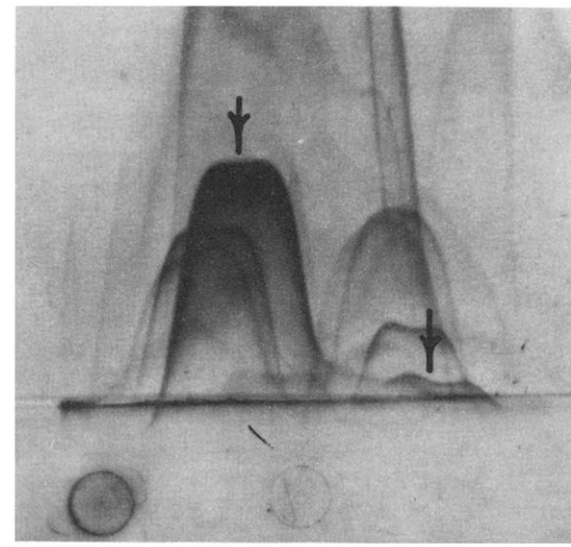

Fig.5. Analysis of a succinate dehydrogenase-negative mutant by tandem crossed immunoelectrophoresis. The left well contained a membrane extract from strain MR43L/F152 and the right well from CBT312 (both 100 μg protein). The extracts were allowed to diffuse for 1 h and then the wells were plugged with agarose. Electrophoresis was at 4 mA for 1.5 h in the first dimension followed by 16 h at 1.5 mA against 80 μl antiserum to the major IEF band in the second dimension. The plate was stained first for succinate dehydrogenase activity and then for protein. The succinate dehydrogenase-specific arc is arrowed.

4. Discussion

4.1. *Multiple succinate dehydrogenase-staining precipitin arcs*

Crossed immunoelectrophoresis of a membrane extract against antiserum raised to isolated inner membrane showed 2 succinate dehydrogenase-staining precipitin arcs. One of these (arc 1) was precipitated also by the antisera raised to the major and minor IEF bands. The other (arc 2) was also precipitated by the antiserum raised to the major IEF band, but the

antibody was present at a much lower titre. It appears, therefore, that arc 1 is the major succinate dehydrogenase-staining arc, and, thus, this arc was further characterized. The nature of arc 2 requires further study.

The presence of 2 succinate dehydrogenase-staining IEF bands also requires further investigation. Both bands gave rise to the same succinate dehydrogenase-specific antibody. It is possible that the 2 bands represent different association states of the enzyme.

4.2. *Properties of succinate dehydrogenase*

The above results indicate that the membrane-bound succinate dehydrogenase of *E. coli* contains non-haem iron and consists of 2 subunits of M_r 73 000 and 26 000. The nature of the other iron-containing antigens was not investigated further. A full description of iron-containing antigens appears in [16]. The presence of non-haem iron is not unexpected since it is found in mitochondrial succinate dehydrogenase [17]. Furthermore, EPR signals have been detected in *E. coli* membranes which are similar to those of mitochondrial succinate dehydrogenase [18].

Comparison of subunit structure with that of the analogous mitochondrial enzyme is complicated by the fact that in mitochondria succinate-dependent reduction of artificial electron acceptors such as phenazine methosulphate involves only 2 subunits of M_r 70 000 and 27 000 [17] while the succinate-dependent reduction of ubiquinone requires two additional subunits of M_r 13 500 and 7000, the 4 subunit complex making up complex II [19]. In this study only the succinate-dependent reduction of phenazine methosulphate was stained for. The involvement of subunits other than the 2 detected in the succinate-dependent reduction of ubiquinone by *E. coli* cannot be ruled out. The succinate dehydrogenase of *Bacillus subtilis* is reported to contain a third subunit, a *b*-type cytochrome [20]. No haem was found in our immunoprecipitate. In [1] a cytochrome-free succinate dehydrogenase from *E. coli* was able to reduce phenazine methosulphate. Although the involvement of a cytochrome in the reduction of ubiquinone cannot be excluded, no such cytochrome is an integral part of *E. coli* succinate dehydrogenase as immunoprecipitated from a Triton X-100-solubilized membrane extract.

The mitochondrial enzyme also contains flavin [17]. A preliminary report [4] indicates that succinate dehydrogenase from *E. coli* also contains flavin. The reported 7 subunit composition of the enzyme [4] was much more complex than that reported here. The reason for the discrepancy is unknown since both studies employed similar methods, although the strains used were different (an ML strain was used [4]). It is significant in our study that the same 2 subunits were obtained with each of the 3 antisera since any contaminating precipitin complex would be different in each case. We thus conclude that the 2 subunits are indeed part of the succinate dehydrogenase and are not contaminants. The presence of the larger subunit was indicated in [21] where a 67 000 M_r protein was reported absent from a succinate dehydrogenase-deficient mutant.

It is still possible that the 2-subunit succinate dehydrogenase in *E. coli* K12 is bound to other subunits within the membrane but that detergent solubilization disrupts these associations. Further work will be required to examine the possibility of specific, but labile, associations which may occur within the membrane.

Acknowledgements

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