Communication

Isolation of Reconstitutively Active Succinate Dehydrogenase in Highly Purified State*

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BRIAN A. C. ACKELL, EDNA B. KEECH, AND CHRISTOPHER J. COLES
From the Department of Biochemistry and Biophysics, University of California, San Francisco and the Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121

SUMMARY

Existing procedures for the isolation of mammalian succinate dehydrogenase yield preparations of high purity or retain reconstitution activity, but not both. A new procedure is described for the isolation in good yield of virtually homogeneous preparations with full reconstitution activity, and retaining iron-sulfur cluster 3 and the "low $K_m$" reaction site of ferricyanide. On reincorporation of the soluble enzyme into alkali-treated membranes, the same high turnover number ($\sim 21,000$ min at $38^\circ$) is obtained in catalytic assays as with intact inner membrane preparations.

When succinate dehydrogenase is removed from its natural environment of the inner mitochondrial membrane, it becomes extremely labile, modifications to its structure being manifested as changes in various activities of the enzyme. In order to investigate more closely what structural modifications may occur and to relate them to changes in catalytic activity, it is highly desirable to have available a soluble form of the enzyme which is essentially pure and as unmodified as possible. Preparations hitherto described do not meet all these criteria.

The first highly purified preparation reported (1) appeared homogeneous by physical criteria, but a major contaminant was later detected on SDS-acrylamide gels. Moreover, it does not restore succinoxidase activity to alkali-treated membranes. King's (2) modification of the 1-butanol extraction procedure (3) yields preparations in which nearly all the catalytically active molecules are also reconstitutively active, but the purity is low ($\sim 30\%$) and further purification inevitably results in the loss of reconstitution activity, since the latter survives only for a short period in the soluble state, even in an aerobiosis. Further, when Keilin-Hartree particles are used as the starting material, as recommended (2), the turnover number in the succinate/PMS/DCIP assay is low ($\sim 10,000$ to $11,000$), apparently because the starting material contains some inactivated enzyme, which is extracted along with the active enzyme and contributes to the histidyl flavin content on which the turnover number is based (4). In order to circumvent this last problem, ETP, which does not contain inactivated succinate dehydrogenase, has been used as the starting material (4). The 1-butanol/succinate-extracted enzyme from this source has a high turnover number ($\sim 15,000$ at $38^\circ$); $85$ to $90\%$ of the enzyme molecules are reconstitutively active; nearly all have low $K_m$ ferricyanide reductase activity; and the enzyme retains the EPR signal attributed to the Fe$S_{\text{h}}$ nonheme iron cluster (HiPIP or center $3$). This signal is not elicited if the enzyme is reconstitutively inactive. The purity of this type of preparation, however, is still low ($\sim 30\%$).

Another useful procedure is that of Davie and Hatofi (5), in which the enzyme is extracted from Complex II by strong perchlorate solutions in the presence of succinate and a thiol. The preparation is essentially pure, but suffers from the fact that its reconstitution activity is low, i.e. only about $15\%$ of the molecules are reported to be capable of recombining with alkali-treated membrane preparations (ETP) and reconstituting succinoxidase activity (6). In confirmation of this, we have found that only about $15\%$ of the enzyme population has low $K_m$ ferricyanide reductase activity (7), another test for the intactness of the enzyme (8, 9).

This communication describes a new method for the rapid isolation of the enzyme which combines the advantages of the King (2) and Davis-Hatefi (5) procedures and yields an enzyme of high purity with essentially full reconstitution activity.

MATERIALS AND METHODS

ETP was prepared (10) and alkali-inactivated (2), as described. Complex II was isolated with minor modifications (11) of the methods of Baginsky and Hatefi (12). The Davis-Hatefi preparation was isolated from Complex II by the procedure of these authors (5) but with meticulous care used to maintain aerobiosis. Reactivity with ferricyanide at the low $K_m$ site (8) and with PMS/DCIP (13) were measured by the standard procedures quoted, except that $20\, \text{mM}$ Tris/sulfate, containing $0.1\, \text{mM}$ EDTA, pH $7.6$ (at $38^\circ$) was substituted for phosphate buffer.

The soluble enzyme preparations were stored as $\left(\text{NH}_4\right)_2\text{SO}_4$ pellets in liquid $N_2$. These were redissolved at $0^\circ$ immediately before use in a solution of $20\, \text{mM}$ succinate, $20\, \text{mM}$ Tris/sulfate, $0.1\, \text{mM}$ EDTA, pH $7.6$, which had been exhaustively bubbled with argon, and the solutions of enzyme were maintained at $4^\circ$ under a stream of $\text{H}_2\text{O}$-saturated argon. Reconstitution was monitored with an O$_2$ electrode at $30^\circ$ by the appearance of succinoxidase activity when soluble enzyme was added to alkali-treated membranes ($0.56\, \text{mg}$) in a medium containing bovine serum albumin ($1.8\, \text{mg}$), EDTA ($1\, \text{mM}$), cytochrome $c$ ($30\, \mu\text{M}$), and $200\, \text{mM}$ succinate, $50\, \text{mM}$ Hepes buffer, pH $7.6$, in a volume of $1.75\, \text{ml}$. The HiPIP center content was determined by EPR after incorporation of the enzyme into alkali-treated ETP (9).

Protein was measured by the biuret method (14) after precipitation with trichloroacetic acid, where necessary, and, in the case of Complex II, prior washing with acetone/HCl. Enzyme concentration was calculated from the histidyl flavin content of the preparation (15).

RESULTS AND DISCUSSION

The dehydrogenase was isolated from Complex II by a procedure based on the steps and apparatus described by King (2) for the purification of the enzyme from Keilin-Hartree particles.
Complex II (usually 100 to 120 mg) was stirred at 10 mg/ml in 50 mM potassium phosphate, 20 mM succinate, 1 mM dithiothreitol, pH 7.6, and kept under a continuous flow of H₂O saturated high purity argon for 45 min at room temperature. Subsequent operations were conducted at 0-2°C. All solutions, 1-butanol, and the suspension of calcium phosphate gel were first bubbled with argon for 20 to 30 min, before being introduced into the anaerobic extraction vessel. The suspension was cooled to 0°C, the pH adjusted to 9.0 with O₂-free 1 N NaOH, and one-fifth the volume of 1-butanol (prechilled to −20°C) was added anaerobically and dropwise, so that no increase in temperature occurred. After a 20-min incubation, the mixture was centrifuged anaerobically in capped tubes at 105,000 x g for 30 min. The clear aqueous layer was transferred to the extraction vessel which was continuously flushed with a stream of H₂O-saturated argon. Ammonium sulfate fractionation of the eluted enzyme was performed with particular attention to the maintenance of anaerobiosis, and the pellets of enzyme were stored in liquid N₂.

Table 1 summarizes the purification procedure. It is seen that the yield is excellent and that, in contrast to the preparation isolated from Keilin-Hartree particles by a very similar procedure, the turnover number in catalytic assays is near 15,000, the value recorded for the best soluble preparations from ETP (4). The SDS-acrylamide electrophoretic pattern reveals only the two subunits of the enzyme. Further evidence for purity is the histidyl flavin content, 0.97 mol/100,000 g in the best preparations. The iron and labile sulfide content are 8 g atoms each/m01 of histidyl flavin.

Table 1

| Stage                              | Specific activity* | Total activity | Yield % | Turnover number* |
|------------------------------------|-------------------|----------------|---------|-----------------|
| Complex II                         | 45.5              | 5,600          | 11,700  |                 |
| After 1-butanol treatment at pH 9   |                   |                |         |                 |
| Aqueous phase                      | 80.6              | 5,300          | 95.0    | 13,300          |
| Residue                            | 2.7               | 152            | 2.7     | 2,700           |
| Adsorption on calcium phosphate gel|                   |                |         |                 |
| Not adsorbed                       | 3.2               | 47             | <1.0    |                 |
| Gel wash                           | 0                 | 0              | 0       |                 |
| Gel eluate                         | 120.0             | 4,420          | 79.0    | 14,600          |

a Micromoles of succinate oxidized/min/mg of protein at 38° in the succinate/PMS/DCIP assay at Vₘₐₓ (13).

b Based on histidyl flavin content of enzyme fraction.

c From data in Ref. 6.

A characteristic feature of preparations in which virtually all enzyme molecules are reconstitutively active is that the low Kₕₐ ferricyanide activity very nearly equals the activity in the PMS/DCIP assay (7). This is true of the present preparation (Table I), whereas in typical Davis-Hatfield preparations it is only ~20% of the PMS/DCIP activity and ~50 to 60% in samples isolated with special precautions to exclude O₂. The EPR-detectable HiPIP content of the enzyme isolated as described here, measured by a new procedure involving incorporation in alkali-treated membrane (9), is 0.5 to 1 mol/mol of flavin, as high as the best values reported (9). Finally, up to 95% of the enzyme is reconstitutively active. This can be seen in the first column of Table II, which compares the amounts of purified enzyme which must be added to an alkali-treated membrane with that originally present in the intact membrane, in order to achieve equal succinoxidase activity.

Table II

| Preparation               | Reconstitution quotient* | Low Kₕₐ ferricyanide reductase activity (turnover number) | Ratio of EPR-detectable HiPIP to incorporated flavin in reconstituted ETP | FMS-reductase activity (turnover number)* |
|---------------------------|--------------------------|----------------------------------------------------------|--------------------------------------------------------------------------|------------------------------------------|
| Present procedure         | 0.92                     | 12,700                                                   | 0.5-1.6                                                                  | 19,100-14,600                            |
| Davis-Hatfield preparation| 1.15                     | 9,700                                                    |                                                                         |                                           |
| Same, prepared in improved anaerobiosis | 0.37 | 7,600 | 12,700 |
| ETP                       | (0.15-0.2) Absent (1.0)  | 20,000-22,000                                           |                                                                         |                                           |

a Nanomoles of enzyme added/mg of alkali-treated ETP to restore original succinoxidase activity.

b Moles of succinate oxidized/min/mol of histidyl flavin at 38°.

c From data in Ref. 6.

**Fig. 1.** Comparison of the reconstitution activities of succinate dehydrogenase extracted from Complex II with perchlorate under strictly anaerobic conditions (○) and by the 1-butanol method (×). Succinoxidase activities were determined under the conditions described under "Material and Methods."
activity. This is based on the type of plot shown in Fig. 1 which compares the enzyme made by the new method with the perchlorate-extracted enzyme isolated with meticulous care for maintaining anaerobiosis. As noted here, the 1-butanol-extracted enzyme has much greater reconstitution activity. On comparison with preparations isolated exactly as described by Davis and Hatefi (5), an even greater (about 7-fold) difference is found (6). If the same data are plotted with the abscissa denoting only the moles of enzyme having low \( K_m \) ferricyanide activity, instead of total enzyme, added to the membrane, then the two types of preparations give nearly coincidental reconstitution activity. This shows once again that the low \( K_m \) ferricyanide reductase and reconstitution activities are interchangeable measures of the intactness of the dehydrogenase (7-9).

It is well known that the turnover number of the enzyme is much higher in mitochondria and in the membrane than in the extracted form, but that reincorporation into the membrane restores the original high turnover number (4). We regard this as an expression of the modulation of the activity by the membrane environment. In untreated membrane samples, the turnover number at 38° is 21,000 to 23,000 in the PMS/DCIP assay (4). Alkali treatment abolishes this activity. On mixing anaerobically, under conditions previously described (4), an alkali-treated ETP containing ~12 nmol of succinate dehydrogenase in the original active state with a substoichiometric amount (4.9 nmol; 62 units of activity) of the highly purified enzyme just described, almost all of the enzyme recombines with the membrane and there is an absolute increase in the total dehydrogenase activity (activity units now equal 86.5 units). The reconstituted membrane, collected by sedimentation, has, within experimental error, the same high turnover number (TN = 21,000) as untreated ETP, while the trace of uncombined enzyme in the supernatant (maintained anaerobic during centrifugation) has a low turnover number in this assay (6,200) and lacks completely low \( K_m \) ferricyanide reductase activity.

The advantage of using Complex II as starting material for preparation of the soluble enzyme is that significant purification has been achieved prior to rendering the enzyme soluble, whereupon it becomes extremely labile. The anaerobic 1-butanol/succinate extraction procedure applied here serves to preserve the extremely labile activity for the few steps then needed to achieve essentially full purification. The data suggest that the lower reconstitution activity of the Davis-Hatefi preparation may arise during extraction of the enzyme with perchlorate. We feel the most deleterious feature of this method may be the introduction of air in the homogenizations used in the perchlorate extraction.

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