RESTORATION OF RECONSTITUTIVE CAPACITY OF SUCCINATE DEHYDROGENASE BY RHODANESE

S. PAGANI, C. CANNELLA, P. CERLETTI, L. PECCI
Department of General Biochemistry, University of Milan and Department of Biological Chemistry, University of Rome, Italy

Received 3 December 1974

1. Introduction

Succinate dehydrogenase (E.C. 1.3.99.1, SD) contains thiol, disulfide and acid labile sulfur, the latter as a complex with iron. The distribution and possible role of thiol and disulfide groups in the flavoprotein and its subunits has been discussed in a previous paper [1]. The system acid labile sulfur-iron is involved in the catalytic activity [2] and in maintaining the flavoprotein in a condition to transfer electrons to other carriers of the respiratory system [3,4].

The iron sulfur system is very labile, and a direct approach has so far not been possible. Circumstantial evidence [3–6] indicates that a measure of its integrity in isolated succinate dehydrogenase is given by the reconstitutive capacity, i.e. the ability of soluble enzyme preparations to restore a functioning oxidase system in sub-mitochondrial particles de-activated by alkali. The labile sulfide content of the flavoprotein is less closely related with the native state of the iron sulfurcenter [3,7,8].

In previous research it was shown that rhodanese (E.C. 2.8.1.1) and thiosulfate may substitute inorganic sulfide in restoring ferredoxin from apoferredoxin in the presence of $Fe(NO_3)_3$. This suggested a possible role of rhodanese in forming labile sulfide from thiosulfate in iron-sulfur proteins [9]. It seemed interesting to study whether also succinate dehydrogenase is influenced by rhodanese. Changes in reconstitutive capacity and in labile sulfide content were followed as signs of modifications involving the iron sulfur center.

2. Materials and methods

Succinate dehydrogenase was purified as previously described [10] from Keilin Hartree heart muscle preparations made according to King (Method 3) [11]. The enzyme was at the DEAE Sephadex eluate stage and was aged enough time to inactivate it partially to reconstitution.

Rhodanese was prepared from beef kidney and was crystallized in the presence of 1 mM thiosulfate [12]. The average sulfur content was 1.5 g atoms per 37 000 g protein. Sulfane sulfur was removed from rhodanese by treatment with excess cyanide followed by filtration on Sephadex 50 fine (de-sulfur rhodanese).

Succinate dehydrogenase was incubated with rhodanese in 30 mM succinate and 50 mM phosphate buffer pH 7.6.

Keilin Hartree heart muscle preparations were inactivated at 37°C and pH 9.3 (ATKH) according to King [5]. Succinoxidase was reconstituted by incubating 5 mg ATKH and 1 mg succinate dehydrogenase (corresponding to 7–8 μmol histidyl FAD) in a volume of 10 ml. The sedimenting particles were separated as per King [5]. The oxidase activity restored in ATKH measures the reconstitutive capacity of soluble succinate dehydrogenase and is expressed as percent of the activity of the untreated Keilin Hartree preparation.

Succinoxidase activity was measured with a Clark oxygen electrode at 30°C on 1–1.5 mg proteins in 3 ml, 40 mM succinate, 25 μM cytochrome *c*, 50 mM phosphate buffer pH 7.8.

North-Holland Publishing Company – Amsterdam

The activity of succinate dehydrogenase was measured spectrophotometrically with 2 mM phenazine methosulphate and 0.8 mM 2,4-dichlorophenol-indophenol [13]; that of rhodanese was followed by the rate of thiocyanate formation from thiosulfate and cyanide, with the method of Sörbo [14].

Acid labile sulfide was determined according to King and Morris [15], proteins with a biuret method [16] and histidyl FAD as previously described [17].

3. Results and discussion

The reconstitutive capacity and the labile sulfide content of purified succinate dehydrogenase rapidly decay, even in anaerobiosis. Rhodanese slows down both phenomena and when added to partially deactivated preparations it raises the reconstitutive capacity above the value measured at the moment of addition (fig.1). The amount of increment depends on the previous history of the flavoprotein. Labile sulfide is protected, but at 2°C not increased above the initial value. Sulfur in rhodanese does not react like labile sulfide and therefore does not interfere with the labile sulfide measured. The optimal molar ratio of succinate dehydrogenase to rhodanese is 3:1.

Thiosulfate, a good sulfur donor to rhodanese, assayed in a concentration range from 0.3 to 30 mM does not significantly modify the effect of rhodanese on the reconstitution capacity of succinate dehydrogenase,

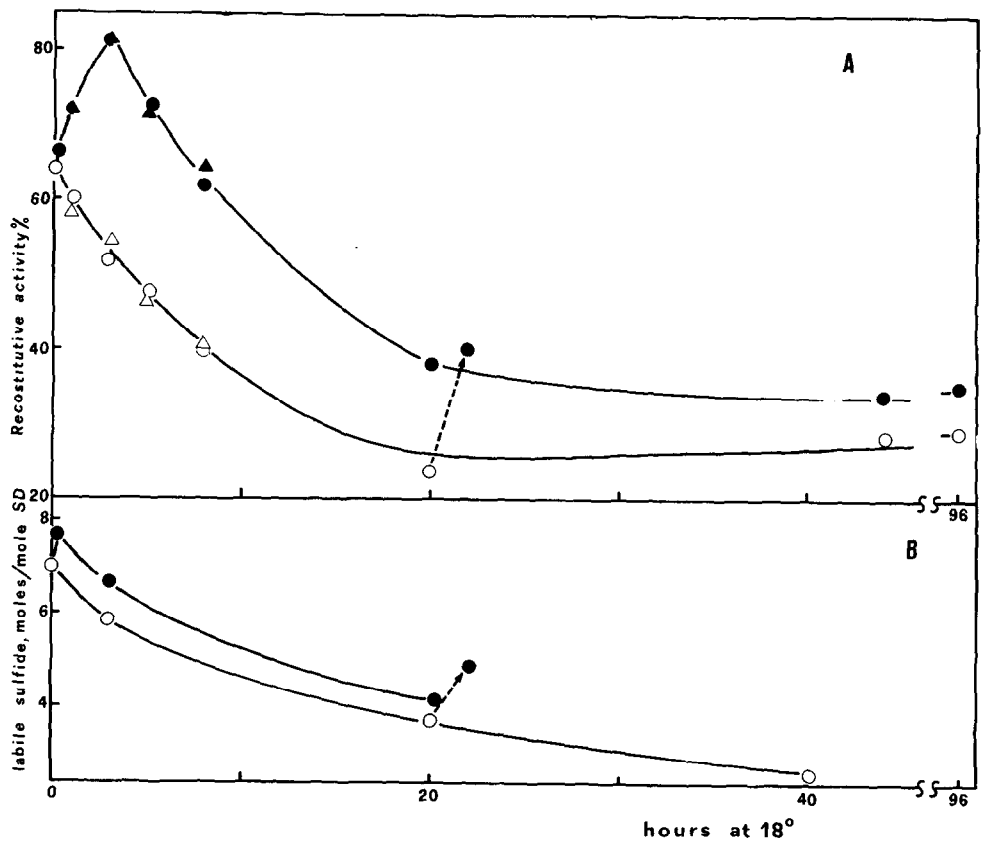

Fig.1. Effect of rhodanese on the reconstitutive capacity (A) and labile sulfide content (B) of succinate dehydrogenase. Succinate dehydrogenase, 9 mg was aged under nitrogen in 40 mM succinate and 50 mM phosphate buffer pH 7.6, without (open symbols) or with (full symbols) 0.55 mg rhodanese, with (triangles) or without (circles) 3.3 mM $Na_2S_2O_3$ in the medium. At the time indicated the reconstitutive capacity and labile sulfide were assayed. The dashed arrow points the change after adding rhodanese to the sample indicated.

nor does it act alone on the flavoprotein (fig.1). Labile sulfide cannot be measured in the presence of thiosulfate. Inorganic sulfide (4 mM Na_2S) with or without 5 mM DTT does not affect succinate dehydrogenase either.

Rhodanese does not restore succinate oxidase in ATKH, but protects moderately (13%) if present during alkaline treatment. It does not modify the catalytic activity of succinate dehydrogenase in phenazine reduction.

Desulfo-rhodanese does not affect the reconstitutive capacity of succinate dehydrogenase. If rhodanese acts together with or after the desulfo-enzyme, its effect on the flavoprotein decreases and this may indicate competition between the two forms of rhodanese. Desulfo-rhodanese treated with thiosulfate to restore the sulfane sulfur in the molecule, has an effect similar to that of rhodanese (table 1).

When succinate dehydrogenase and rhodanese are centrifuged together on a density gradient, the dehydrogenase sediments faster, and the peak of rhodanese has a smaller area than that observed when the two enzymes are centrifuged separately (fig.2). This suggests rhodanese forms a complex with the flavoprotein. No rhodanese activity is detected in the peak of succinate dehydrogenase: this may be rationalized by supposing that, when rhodanese is associated with the flavoprotein, it has no activity in the formation of thiocyanate from thiosulfate. Indeed, rhodanese activity, when measured in the presence of succinate dehydrogenase, decreases considerably.

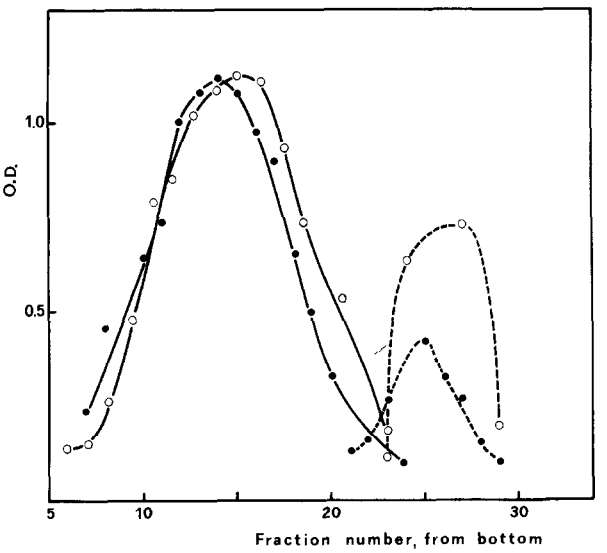

Fig.2. Sedimentation of succinate dehydrogenase (0.45 mg) and of rhodanese (45 μg) alone (open symbols) or in a mixture (full symbols), in a 5 to 25% linear glycerol gradient in 20 mM succinate and 50 mM phosphate, pH 7.6. The tubes, each containing 5.1 ml, were spun 16 hr at 45 000 rev/min in a Spinco L2 65B ultracentrifuge using a SW 65 Ti rotor. At the end of the run, 36 fractions were collected from each tube. Solid line SD activity. Dashed line, rhodanese activity. Abscissa: fraction number from the bottom of the tube.

Table 1
Effect of de-sulfurated rhodanese on succinate dehydrogenase

Additions	Incubation, hr 1	2
None	43.0	37.0
Rhodanese	56.0	47.0
De-sulfur rhodanese	41.5	37.0
De-sulfur rhodanese + $Na_2S_2O_3$	46.0	
De-sulfur rhodanese pretreated with $Na_2S_2O_3$*		44.0**
Rhodanese + de-sulfur rhodanese	50.0	
De-sulfur rhodanese, 1 hr, then rhodanese		39.0**

* Pretreatment 1 hr at 2°C. Then mixed with succinate dehydrogenase aged 1 hr at 2°C.

** Total aging of SD before reconstitution, 2 hr.

Succinate dehydrogenase, before reconstitution, was incubated at 2°C in the conditions indicated. Rhodanese and de-sulfur rhodanese were 1 mg per 10 mg flavoprotein. $Na_2S_2O_3$ was 30 mM. The percent reconstitution is given.

The above results indicate that rhodanese interacts with succinate dehydrogenase inducing changes which may be related to modifications in the iron sulfur center. Sulfane sulfur is required in the molecule for rhodanese to interact correctly with the flavoprotein. Moreover the active center of interacting rhodanese is not available to external thiosulfate. In this respect the action of rhodanese on succinate dehydrogenase differs from that on apoferredoxin, where the effect is supported by added thiosulfate [9].

Since external thiosulfate does not affect the efficiency of rhodanese action on the flavoprotein, either the phenomena described do not involve any overall quantitative change of labile sulfide, or there is an internal supply of sulfur to rhodanese. In the first alternative, since reconstitutively inactive succin-

ate dehydrogenase still contains labile sulfide, in the presence of rhodanese a rearrangement may occur involving this species of sulfur, restoring reconstitutively active molecules. The other alternative is supported by the finding by Petering and Palmer that when the iron–sulfur system is degraded under oxidizing conditions, sulfer is bound to the protein molecule as a trisulfide [18]. Trisulfide are sulfur donors to rhodanese [19] and in the present case, trisulfide sulfur formed from labile sulfide in deactivated molecules of succinate dehydrogenase might be the species exchanged. Although in our experiments anaerobiosis was strictly controlled some access of air may have occured during the manipulations. Moreover limited internal oxidation might be supported by Fe^{III} in the molecule of succinate dehydrogenase. The fate of iron in the phenomena reported, and in our experiments has never been investigated and may be the object of further research.

Acknowledgement

The present study was supported by a grant of the Italian National Research Council.

References

[1] Pagani, S., Bonomi, F. and Cerletti, P. (1974) FEBS Lett. 39, 139–143.
[2] Dervartanian, D. V., Veeger, C., Orme Johnson, W. H. and Beinert, H. (1969) Biochim. Biophys. Acta 191, 22–37.
[3] Baginsky, M. L. and Hatefi, Y. (1969) J. Biol. Chem. 244, 5313–5319.
[4] Hanstein, W. G., Davis, K. A., Ghalambor, M. A. and Hatefi, Y. (1971) Biochemistry 10, 2517–2524.
[5] King, T. E. (1963) J. Biol. Chem. 238, 4037–4051.
[6] Kimura, T. and Hauber, J. (1963) Biochem. Biophys. Res. Commun. 13, 169–174.
[7] King, T. E. (1961) Biochem. Biophys. Res. Commun. 16, 511–515.
[8] Bernath, P., Singer, T. P. (1962) in: Methods in Enzymology (Colowick, S. P., Kaplan, N. O., eds.), vol. 5, p.597, Academic Press, New York.
[9] Pinazzi Agrò, A., Cannella, C., Graziani, M. T. and Cavallini, D. (1971) FEBS Lett. 16, 172–174.
[10] Cerletti, P., Zanetti, G., Testolin, G., Rossi, C., Rossi, F. and Osenga, G. (1971) in: Flavins and flavoproteins (Kamin, H., ed.), p.629, University Park Press, Baltimore.
[11] King, T. E. (1967) in: Methods in Enzymology (Estabrook, R. W., Pullman, M. E., eds.), vol. 10, p.202, Academic Press, New York.
[12] Cannella, C., Pecci, L. and Federici, G. (1972) Italian J. Biochem. 21, 1–7.
[13] Cerletti, P., Giovenco, M. A., Giordano, M. G., Giovenco, S. and Strom, R. (1967) Biochim. Biophys. Acta 146, 380–396.
[14] Sörbo, B. H. (1953) Acta Chem. Scand. 7, 1129–1136.
[15] King, T. E. and Morris, R. O. (1967) in: Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), vol. X, p.634, Academic Press, New York.
[16] Itzhaki, R. F. and Gill, O. M. (1964) Anal. Biochem. 9, 401–410.
[17] Cerletti, P. and Giordano, M. G. (1971) in: Methods in Enzymology (McCormick, D. and Wright, eds.), vol. 18B, p.285, Academic Press, New York.
[18] Petering, D., Fee, J. A. and Palmer, G. (1971) J. Biol. Chem. 246, 634–653.
[19] Westley, J. (1973) Advances Enzymol. 39, 327–368.