SULFHYDRYL AND DISULFIDE CONTENT OF SUCCINATE DEHYDROGENASE*

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1. Introduction

Succinate dehydrogenase (SD, EC 1.3.99.1) has been purified in our and in Hatefi's laboratories [1, 2] and its protomer structure was established. The enzyme is made of two unlike subunits, of mol.wt. 69 000 and 30 000 respectively in a 1:1 ratio. Both contain non heme iron and labile sulfide [2] but only the larger one contains peptide bound flavin (PBF) [1, 2].

In our laboratory it was shown that no stable bond links the subunits and they can be resolved under non reducing conditions. Moreover the fact that *p*-hydroxymercuribenzoate does not affect the association, suggests that no sulfhydryl groups are implicated in linking the subunits [3]. The association is probably governed by hydrophobic forces: indeed low temperatures favour the dissociation between sununits and the flavoprotein is not resolved when treatment with chaotropes is combined with heating [4]. This type of temperature dependence is typical of hydrophobic forces.

It is known that groups reacting with mercurials are important for the catalytic behaviour and stability of SD. The available evidence indicates that the iron-labile sulfide structure is important for the reconstitution capacity and that sulfhydryl (SH) groups are involved in the activity towards artificial electron acceptors [5–7]. It has been suggested that the same SH group takes part in the first step of succinate oxidation, in the inactivation of SD in the presence of oxygen and in the irreversible binding of oxaloacetate [7, 8]. Sulfhydryls are also involved with the inactivation by *N*-ethylmaleimide relieved by malonate [9].

However both SH and labile sulfide interact with mercurials and the interpretation of results is not straightforward unless separate estimation of the effects on either grouping are made. So far no quantitative data on the SH content of SD are available and only the amount of labile sulfide (8 moles per mole PBF) is known.

In this communication we report the content of the enzyme in SH and disulfide (S–S) groups and their reactivity under different conditions. This study allowed further understanding of the enzyme, on the structure of its subunits and their interactions in the molecule.

2. Materials and methods

Chemicals were from Merck & Co. and from DBH. Non heme iron was determined according to Doeg and Ziegler [10], peptide bound flavins as previously described [11], proteins with a biuret method [12] and succinate dehydrogenase activity spectrohotometrically with 2 mM phenazine methosulfate and 0.8 mM 2,4-dichlorophenol-indophenol as sequential acceptors [13].

Sulfhydryl groups were titrated amperometrically using the phenyl-mercury acetate as primary standard and a dropping mercury electrode, according to Petering and Palmer [14], due allowance being made

* Paper V in this series on succinate dehydrogenase see ref. [1].

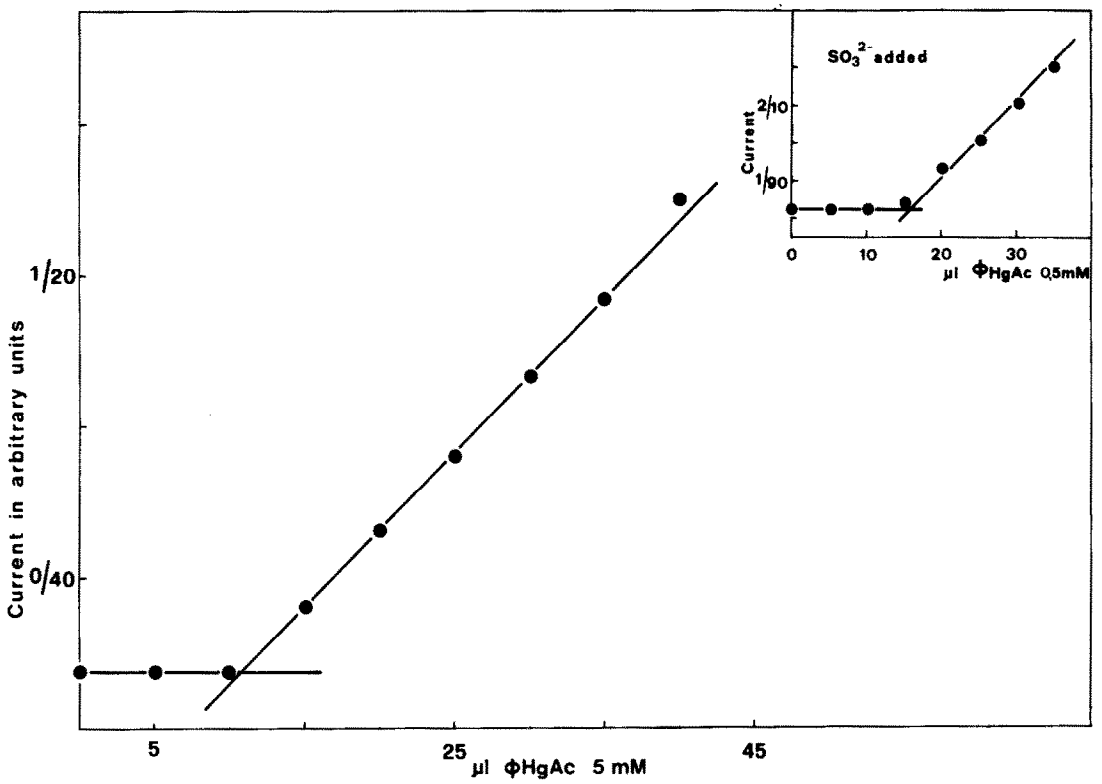

Fig. 1. Sulfhydryl and disulfide titration in succinate dehydrogenase. The enzyme was purified by electrophoresis on a Uniphor column, and was incubated (1.56 mg protein/ml) with 6 M guanidine hydrochloride for 1 hr at 0°C in 20 mM Tris–HCl buffer pH 7.60. It was titrated in this medium with 5 mM phenyl-mercury acetate [14]. 3 mM Na_2SO_3 was then added, the mixture kept 40 min at room temperature and then titration resumed with 0.5 mM phenyl-mercury acetate.

for the content in labile sulfide. Labile sulfide was determined according to King and Morris [15] on a separate aliquot of the sample, and its content was subtracted from the results of the amperometric titration. Since SD easily loses labile sulfide, the correct evaluation of labile sulfide in the very conditions in which SH groups are titrated is essential for exactly estimating them. Disulfide bonds were determined after splitting the bond with SO_3^{2-} [14].

Sulfhydryl and S–S groups were titrated, in parallel with peptide bound flavin and protein determinations, in the holoprotein and in the isolated heavier subunit. The data obtained on these preparations served to calculate the content in both protomers.

Beef heart SD was used after elution from DEAE-Sephadex, a preparation which contains about 15% impurities [16]. This preparation is fully catalytically and reconstitutively active. As a check we also titrated SD, obtained after further purification by electrophoresis on polyacrylamide in a Uniphor column (LKB 7900)[17]. This preparation is homogeneous as indicated by molecular weight determination on peptide bound flavin basis, by ultracentrifugation, zone and sodium dodecyl sulphate (SDS) electrophoresis and other criteria [3], but is in part catalytically denatured during the preparative treatments. Modifications in exposure of SH groups due to various treatments were measured on the DEAE-Sephadex eluate.

The subunits were separated by freezing and thawing purified SD in 0.4 M sodium trichloroacetate [2]. This treatment precipitates the heavier subunit with a 15% impurity of the lighter one. The lighter subunit remains in the supernatant but is heavily contaminated, as SDS electrophoresis shows, by the heavy one.

The purity of each preparation of enzyme and subunit was routinely checked by peptide bound flavin

Table 1
Sulfhydryl content of succinate dehydrogenase under different conditions.

Pretreatment of enzyme	Conditions at titration	SH groups per mole PBF	Standard error	Number of experiments
None	20 mM Tris–HCl buffer pH 7.6	20.17	1.18	3
	60 mM Phosphate buffer pH 8.0	21.60	0.50	3
	Same + 38 mM succinate	20.33	1.01	3
8 M Urea 1 hr, 0°C	0.26 M Urea	20.85	0.60	2
6 M Gu-HCl 1 hr, 0°C	6 M Gu-HCl	19.72	1.12	4

Analysis of variance indicates that differences between experimental groups are not statistically significant. Succinate dehydrogenase was eluted from DEAE-Sephadex G 25 [16] and was titrated anaerobically with 5 mM phenyl-mercury acetate according to Petering and Palmer [14] in the medium shown. Before titration the preparation was treated as stated.

and protein analysis and by SDS electrophoresis and densitometry of the separated bands [1].

All the experiments and titrations were carried out under strict anaerobiosis.

Statistics and analysis of variance were done according to Lison [18].

3. Results and discussion

Fig. 1 shows the titration of SH and S–S groups in SD. In the latter assay the titratant was diluted to increase sensitivity. Both titrations, either before or after SO_3^{2-} addition, have the same slope. The subunits were titrated in the conditions detailed in fig. 1 with 2.0 mM and 0.2 mM phenyl-mercury acetate respectively before and after Na_2SO_3 addition. Clear end-points were obtained in all titrations.

The number of SH groups determined in SD under different experimental conditions is given in table 1. An analysis of variance between the various experimental sets of data does not give any statistical significance to the small differences existing. Activating agents, such as succinate and phosphate, are supposed to modify the conformation of the enzyme [19]. Urea and guanidine hydrochloride denature it. However the number of SH groups does not significantly change. It appears therefore that in the native molecule of SD all SH groups are exposed and freely available to the titratant and binding of succinate does not induce a conformational change in SD in such a manner that SH groups become hidden. Presence of succinate protects the reactive site for substrate, which is proposed to be a SH group [7], from *p*-chloromercuribenzoate added up to 8–10 moles/mole PBF [6]. Further addition allows a complete titration of SH probably because of disruption of the iron-labile sulfide structure.

Disulfide groups on the other hand are titrated only in highly denaturing conditions such as 6 N guanidine hydrochloride.

As a further check to the realiability of the data the enzyme eluted from DEAE-Sephadex was titrated after precipitation with 10% trichloroacetic acid, which removes practically all labile sulfide. The number of SH and S–S determined was the same as in non denatured preparations and in analytically homogeneous preparations of SD electrophoresed on Uniphor (table 2). This was confirmed by analysis of variance.

The quantitative results concerning the holoprotein and the subunits are summarized in table 2. They allow to assign 20±1 SH groups to SD of which 12 are in the heavier protomer and 8 in the lighter one. If we assume for the iron–sulphur center a similar structure to that described for ferredoxin by Dunham et al. [20] the values found allow two such centers in each subunit of SD, in agreement with the content of iron and labile sulfide.

Table 2
Sulphydryls and disulfide of succinate dehydrogenase and its subunits.

	SH groups per mole PBF	Standard error	Number of experiments	S–S groups per mole PBF	Standard error	Number of experiments
Holoprotein:						
Enzyme DEAE	20.70	0.70	13	2.47	0.90	5
Enzyme Uniphor	19.58	0.55	3	3.09	0.08	3
Enzyme DEAE precipitated with TCA	21.50	0.16	2	2.69	0.00	2
Subunit mol.wt. 69 000:						
Experimental results	15.28	0.22	4	3.00	0.47	4
Calculated	12.01	0.53		3.00		
Subunit mol.wt. 30 000:						
Calculated	7.60	0.53		0.00		

Analysis of variance indicates that differences between experimental groups of the holoprotein are not statistically significant. Succinate dehydrogenase was eluted from DEAE-Sephadex (enzyme DEAE) [4] or was also electrophoresed and eluted from Uniphor (enzyme Uniphor) [3]. It was titrated with phenyl-mercury acetate [14] as such or after precipitation with trichloroacetic acid (TCA) or its subunits were first separated [2]. From the experimental results on the holoprotein and on each isolated preparation of heavier subunit the sulfhydryl and disulfide content of both protomers was also algebrically computed.

The disulfide groups are 3 and are all contained in the heavier subunit. The subunit do not interact through disulfide bridges.

Since SD contains Fe III in the molecule [21] a possible source of error in these studies is the oxidation of labile sulfide or of sulfhydryls by Fe III in the flavoprotein. It might produce a disulfide and according to Petering et al. [22] eventually build up with sulfur 'zero' a trisulfide. The artefactual increase of S–S content, if due to SH oxidation, should correspond to a decrease of SH, which is not observed. Only minor difference appear in the titration of preparations of increasing degree of preparative damage (Uniphor enzyme, subunits) and they may be attributed to such a mechanism. A definitive answer will come when Fe III and Fe II will be estimated in native SD and after SH titration.

Acknowledgements

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