INSERTION OF SULFIDE INTO FERREDOXINS CATALYZED BY RHODANESE

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1. Introduction

In previous work [1] we studied the interaction of rhodanese (EC 2.8.1.1) an enzyme present in mitochondria, chloroplasts and bacteria, with the iron–sulfur flavoprotein succinate dehydrogenase (EC 1.3.99.1). Rhodanese catalyzes the transfer of sulfane sulfur from a sulfane containing anion to a thiophilic anion. Intermediate in this reaction is sulfur-substituted rhodanese ([S] rhodanese) which can be isolated. When some dithiol-containing compounds serve as acceptors, e.g., lipoate or dithiothreitol, the persulfide formed by rhodanese breaks down in an internal redox reaction yielding a disulfide and inorganic sulfide [2]. We found that the sulfane sulfur of [^{35}S]rhodanese or that of thiosulfate was transferred to succinate dehydrogenase which bound it as sulfide and had its iron–sulfur structure modified. In a comparative assay with other proteins the presence of the iron–sulfur structure appeared important for sulfide binding [1].

In the present work we have addressed ourselves to the problem of whether reduction and binding of sulfur was peculiar to succinate dehydrogenase or whether it also occurred with other iron–sulfur proteins. The reaction mechanism was also investigated. Since succinate dehydrogenase contains both a Fe_4S_4 structure of the Hipip type and two spinach ferredoxin-like Fe_2S_2 centers [3,4], we assayed spinach ferredoxin and ferredoxin from *Clostridium pasteurianum* which contains two Fe_4S_4 clusters per molecule.

2. Experimental

Spinach ferredoxin was prepared according to [5], clostridial ferredoxin was purchased from Sigma, rhodanese was isolated from beef kidney according to [6].

Incubations were carried out in anaerobic cuvettes in a nitrogen atmosphere. The reacted mixture was resolved on columns of Ultrogel AcA 54 (24 × 2.3 cm) for spinach ferredoxin or of Biogel P 10 (30 × 2.4 cm) for the clostridial ferredoxin. Both types of mixtures were resolved also using Sephadex G-25 (11 × 1.2 cm) followed by DE 52 cellulose (0.8 × 1.0 cm): spinach ferredoxin was eluted from the ion exchanger with 0.15 M Tris-HCl buffer, pH 7.8, containing 0.13 M NaCl; the Tris buffer was 0.1 M, pH 8.1, and NaCl was 0.2 M for the clostridial ferredoxin.

Acid-labile sulfide content was determined by a modified procedure [7]. After spectrophotometric assay the dye was extracted with butanol as described [1]. In samples containing thiosulfate or dithiothreitol the ZnS precipitate from inorganic and protein bound sulfide was separated by centrifugation and transformed into methylene blue according to [8]. Protein bound sulfane sulfur was assayed as sulfide after reduction with excess dithiothreitol according to [9].

All other materials and assay procedures were those described [1].

3. Results

The analytical pattern after incubation of spinach ferredoxin with rhodanese is shown in table 1. Rhodanese protected labile sulfide from decay. When thiosulfate was also present during incubation, total labile sulfide was even increased: ^{35}S incorporated from thiosulfate accounts for the increase. Rhodanese became labelled in part (table 1C). In the absence of

Table 1
Action of rhodanese on spinach ferredoxin

	min	S^{2-} in Fd (nmol. mg^{-1})		^{35}S (mol. mol^{-1})		$^{35}S^{2-}$ (% ^{35}S)		Rhodanese activity
		−Rd	+Rd	in Fd	in Rd	in Fd	in Rd	
A	0	149		0	0.62		0	100
	60	97	141[a]			26.4[a]		87[a]
	200							81[a]
	200 (after AcA 54)		89	0.01	0.10	89.5	0	33
B	0	116		0	1.03			100
	60	94	109[a]			21.7[a]		82[a]
	120							80[a]
	120 (G 25 + DE 52)		98	0.45	0.36	41.2	24.3[b]	50
C	0	144		0	0			100
	60	99	164[a]					100[a]
	190							100[a]
	190 (after AcA 54)		125	0.23	0.39			73

[a]Unresolved reaction mixture

[b]No methylene blue was produced. The amount of sulfur measured is below the sensitivity of the Fogo procedure. Possible contributions of (labelled) sulfane sulfur from rhodanese, extracted in butanol, are deducted

Ferredoxin (1.44 mg. ml^{-1}) was incubated 60 min without or with rhodanese in 3:1 molar ratio, in 0.1 M Tris–HCl buffer, pH 8.1, at 10°C. In A and B rhodanese was labelled with ^{35}S, in C it had no label and 1 mM $Na_2{}^{35}SO_3$ was added. The reacted mixture was in part gel filtered at 2°C, the rest being stored for an equivalent time. In B the sample before gel filtration received 0.9 mM $Na_2{}^{35}SO_3$. Sulfide, sulfane sulfur, radioactivity, and labelled sulfide were assayed in the reaction mixture, on the separated proteins and in the low molecular weight compounds. Rhodanese activity in thiocyanate formation is expressed as percent of a control without ferredoxin, treated as the sample. Fd, ferredoxin; Rd, rhodanese

the transferase ferredoxin did not incorporate sulfur from thiosulfate. If labelled thiosulfate was added to the reacted mixture of [^{35}S]rhodanese and ferredoxin before gel filtration (table 1B), labile sulfide of ferredoxin decayed less during chromatography and the protein appeared to have bound more ^{35}S, part of it however not as sulfide. On the other hand some sulfur in rhodanese reacted as sulfide.

The sulfur transferase was deactivated in the reaction, but thiosulfate prevented this effect. Reacted rhodanese became quite labile when it was separated from the other reactants, however the presence of thiosulfate in the reaction medium or added before gel filtration reduced the lability (table 2B and C). We have also noticed in other experiments (unpublished) where the sulfide content of ferredoxin was less protected that rhodanese was inactivated less.

Clostridial ferredoxin was a preparation of commercial origin which had lost much of its native labile sulfide. Part of the lost sulfide was bound to the protein as sulfane sulfur (as was the case also for spinach ferredoxin). The residual labile sulfide did not decay significantly in the incubation conditions (table 2). In the presence of [S] -rhodanese alone or with thiosulfate, labile sulfide increased conspicuously. This continued during gel filtration and some sulfide was found also with low molecular weight compounds.

Only 16% of the label released by [S] rhodanese was incorporated into ferredoxin (table 2A): all ^{35}S inserted was sulfide, but it accounts for only 1.5% of the increase in total labile sulfide: added to inorganic [^{35}S]sulfide, which probably has been liberated from ferredoxin during gel filtration, it makes up 2.4% of total sulfide increase in ferredoxin. The

Table 2
Action of rhodanese on clostridial ferredoxin

	min	S^{2-} in Fd (nmol. mg^{-1})		^{35}S (mol. mol^{-1})		$^{35}S^{2-}$ (% ^{35}S)		Rhodanese activity
		-Rd	+Rd	in Fd	in Rd	in Fd	in Rd	
A	0		233	0	1.03		0	100
	60	220	526[a]					90[a]
	150							88[a]
	150 (after P 10)		579[c]	0.03	0.11	97.0	37.3[b]	43
C	0		468	0	0			100
	60	451	557[a]			46.1[d]		69[a]
	300							107[a]
	300 (G 25 + DE 52)		564	0.13	0.88	31.4	9.2[b]	96

[a]Unresolved reaction mixture
[b]Same as [b] table 1
[c]In the gel retarded compounds another 56 nmol. mg^{-1}, of which 5.4% is radioactive
[d]Reacted mixture after removing thiosulfate according to [8]

Ferredoxin (0.34 mg. ml^{-1}) was incubated without or with rhodanese in 0.1 M Tris–HCl buffer, pH 7.3. Incubations and analyses were as in table 1, A and C

restored sulfide accounts for nearly 50% of the initial ferredoxin bound sulfane sulfur.

With thiosulfate in the medium (table 2C), more ^{35}S was incorporated: however only part of it was sulfide and this accounts but for 7.2% of the total increase in labile sulfide. Rhodanese was heavily loaded with ^{35}S. Some of the sulfur in the enzyme incubated with or without thiosulfate behaved as sulfide.

The transferase was less deactivated in the reaction with clostridial than with spinach ferredoxin, and the isolated reacted enzyme was more stable. Thiosulfate in the medium enhanced the decay during reaction (probably a more active interaction), but made the protein less labile when separated from the other reactants (table 2C). As observed with spinach ferredoxin less inactivation occurred when the sulfide content of the iron sulfur protein was less protected.

4. Discussion

Rhodanese markedly protected ferredoxins from loss of sulfide and in proper conditions it even increased the sulfide content, showing that 'protection' is the balance between insertion by rhodanese and decay.

Much more sulfur was transferred to ferredoxins per mole and per mg protein than to succinate dehydrogenase. Some of the sulfide formed did not originate from external sulfur supplied as [S]rhodanese or as thiosulfate: the increase of total sulfide in clostridial ferredoxin exceeded many times the measured bound (labelled) sulfur derived from the transferase or from thiosulfate. Also, in experiments where sulfur-substituted rhodanese was the only external sulfur donor, the measured increase in total sulfide was larger than the amount of sulfur that the enzyme carried into the reaction mixture. The other possible source of sulfur in the reaction is ferredoxin-bound sulfane sulfur which, as shown [9], and confirmed in our data, results from decayed iron sulfur structures.

This unlabelled sulfur if used dilutes the ^{35}S supplied by thiosulfate or by [^{35}S]rhodanese. It also competes with ^{35}S from thiosulfate in forming the sulfur-substituted rhodanese intermediate: indeed (unlabelled) rhodanese reacted with spinach ferredoxin appeared not to incorporate as much sulfur from thiosulfate as it could (up to 1 mol/mol) and as it does, for example, with succinate dehydrogenase [1]. It is interesting that this dilution did not occur with

clostridial ferredoxin, where the impact of ferredoxin-bound sulfane sulfur is most evident and where the (labelled) sulfur of rhodanese was diluted in sulfide formation.

Only part of the (labelled) sulfur transferred was reduced when the incorporation was high. The rest is probably bound in a manner similar to the endogenous sulfane which originated from decayed iron sulfur clusters.

With both ferredoxins rhodanese was inactivated in the early period of reaction. The decay did not continue during storage at 2°C and in one case (table 2C) the ferredoxin-containing sample was inactivated less than the control. Ferredoxins differ in their effect on the catalytic efficiency of rhodanese; each reproduces some facet observed with succinate dehydrogenase [1]. The transferase was severely damaged in the interaction with spinach ferredoxin, as it is with the flavoprotein; with clostridial ferredoxin thiosulfate in the medium enhanced inactivation of rhodanese during reaction, and with both ferredoxins it made the reacted transferase less labile to chromatography.

Rhodanese probably interacts with ferredoxins also during gel filtration, specially if thiosulfate or protein-bound sulfane sulfur are available. However in these conditions the substrate is at least in part modified; indeed on the column rhodanese (mol. wt 37 000) preferentially finds oxidized dimers (mol. wt 24 000 for spinach ferredoxin and mol. wt 12 000 for the clostridial one) or oligomers originated by intermolecular disulfide bonds formed, as described [9], in the decay of ferredoxin.

We are inclined to thank that, either because of molecular shape or because of the oxidation state of sulfur in the ferredoxin molecule, in the above situation reduction of the transferred sulfane occurs predominantly through oxidation of the sulfhydryl groups of rhodanese, as is the case with succinate dehydrogenase [1]. The transferase is thereby inactivated. On the other hand in the unresolved reaction mixture the reduction of sulfane may involve, in a pattern similar to lipoate oxidation by rhodanese, the thiol groups made available in the ferredoxin by the decay of iron sulfur clusters. (Native spinach ferredoxin has one SH per molecule, the clostridial ferredoxin has none.)

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