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A method for the direct recording of an enzyme-catalyzed thiol–disulfide interchange using a mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol

We have recently tried to study the kinetics of a partially purified enzyme from rat liver catalyzing the thiol–disulfide interchange between glutathione and the mixed disulfide of cysteine and glutathione[1]:

$$GSH + CySSG \rightarrow CySH + GSSG \quad (1)$$

The activity was measured as the oxidation of NADPH by the glutathione disulfide formed in a system containing glutathione reductase (*cf.* refs. 2–4). However, it was found that glutathione reductase had a low activity with the mixed disulfide of cysteine and glutathione (about 1% of that obtained with glutathione disulfide). Furthermore, it was apparently also catalyzing the thiol–disulfide interchange itself, as it was not possible to make the overall activity observed independent of the glutathione reductase concentration. Although these complications could be accounted for by control experiments, it was not possible to ascertain that the observed kinetic behavior of the enzyme catalyzing the thiol–disulfide interchange was not influenced by the components of the glutathione reductase system. It was therefore desirable to develop a method for the direct observation of the thiol–disulfide interchange. Such a method could utilize a reaction releasing a colored product from a colorless disulfide, a well-known example of which is the reaction of 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) with thiols[5].

The reaction of DTNB with GSH is actually catalyzed by the partially purified enzyme from rat liver, as is the reaction of DTNB with cysteine (Table I). The catalytic effect, expressed as the ratio of enzymatic to nonenzymatic reaction, differs in these two cases by a factor of more than 20, which demonstrates that the enzyme has a marked specificity with respect to the thiol. However, it was considered more useful to study a mixed disulfide such as that of glutathione and 3-carboxy-4-nitrobenzenethiol ("reduced DTNB"), as we wanted to measure activities with specificity for a GSH sulfenyl group of the disulfide substrate. Furthermore, the kinetics of a reaction between a thiol (R_1SH) and an alkyl-aryl disulfide ($ArSSR_2$) is simpler, as the fission of the S–S bond specifically liberates the arene thiol (ArSH)[6]

$$R_1SH + ArSSR_2 \rightarrow R_1SSR_2 + ArSH \quad (2)$$

The further reactions of R_1SSR_2, an alkyl-alkyl disulfide, will not be observed in this case, whereas the corresponding product of the reaction of a thiol with a symmetrical aromatic disulfide (such as DTNB)

$$R_1SH + ArSSAr \rightarrow R_1SSAr + ArSH \quad (3)$$

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoate).

will be an alkyl-aryl disulfide which reacts according to Eqn. 2 with concomitant spectral changes. Thus, in the latter case the sum of the primary and secondary reactions will be recorded.

The mixed disulfide of GSH and 3-carboxy-4-nitrobenzenethiol was synthesized[7] and was then used to determine the activity of *S*-nucleophiles as substrates for the enzyme catalyzing thiol–disulfide interchange (Table I). The enzyme was a partially purified preparation essentially free from glutathione reductase[8]. It is evident that

TABLE I

COMPARISON OF THE ACTIVITY OF VARIOUS *S*-NUCLEOPHILES AS SUBSTRATES FOR THE ENZYME CATALYZING THIOL–DISULFIDE INTERCHANGE WITH DTNB (A) AND THE MIXED DISULFIDE OF GSH AND 3-CARBOXY-4-NITROBENZENETHIOL (B) AS DISULFIDE SUBSTRATES

Activity was determined in 0.18 M sodium phosphate buffer of pH 5.5 (containing 1 mM EDT and was measured spectrophotometrically as the formation of 3-carboxy-4-nitrobenzenethiolate at 412 nm ($\varepsilon = 12.9\ mM^{-1} \cdot cm^{-1}$). The nonenzymatic reaction was determined in the same system in the absence of enzyme.

Disulfide (40 μM)	*Nucleophile (100 μM)*	*Enzymatic activity (nmoles · min⁻¹)*	*Nonenzymatic reaction (nmoles · min⁻¹)*	*Ratio of enzymatic to nonenzymatic reaction*
A	GSH	29.8	5.84	5.1
	Cysteine	2.66	12.3	0.22
B	GSH	3.40	1.45	2.3
	Cysteine	2.02	2.88	0.70
	Cysteamine	1.39	6.61	0.21
	Penicillamine	0.55	0.84	0.65
	Mercaptoethanol	0.131	0.692	0.19
	3-Mercaptopropionate	0.458	0.249	1.8
	Thioglycolate	7.20	1.40	5.3
	Na_2SO_3	3.82	3.26	1.2
	Na_2S	1.24	1.05	1.2
	$Na_2S_2O_3$	0.328	0.592	0.55
	KSCN	0.0169	0.126	0.13
	KCN	0.0013	0.0080	0.16

this method to determine the activity allows, for the first time, the direct recording of a reaction between a mixed disulfide of glutathione and a thiol different from glutathione.

$$GSSAr + R_1SH \rightarrow GSSR_1 + ArSH \qquad (4)$$

It is noteworthy that cysteine and cysteamine, which are the most reactive thiols, as demonstrated by the rates of their spontaneous reactions, demonstrate low ratios of catalyzed/uncatalyzed rates (Table I). One could consider the possibility that the catalytic effect is due to facilitation of ionization, and that the reactive thiols are ionized to a greater extent than the others, therefore being less affected by the enzyme. However, the pK_a values for cysteine and cysteamine are three units above the pH of the assay system (pH 5.5), and the thiols are therefore essentially completely protonated. The explanation of the differences in enzymatic activity has consequently to be sought in the properties of the enzyme.

We have also tested other *S*-nucleophiles such as sulfite, sulfide, thiosulfate,

thiocyanate and cyanide as substrates for the enzyme (Table I). The activity with sulfite gives support to the view that the reversible reaction between thiols and thiosulfate esters ($RSSO_3^-$)

$$R_1SH + RSSO_3^- \rightleftharpoons RSSR_1 + HSO_3^- \quad (5)$$

is catalyzed by enzymes *in vivo*[9].

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