The effect of 2,4,6-trinitrobenzenesulfonate on mercuric reductase, glutathione reductase and lipoamide dehydrogenase

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Among the three closely related enzymes, lipoamide dehydrogenase, mercuric reductase, and glutathione reductase only the latter is inhibited by 2,4,6-trinitrobenzenesulfonate (TNBS) On the other hand, all three enzymes exhibit high rates of TNBS-dependent NADPH oxidation In the case of glutathione reductase and mercuric reductase this TNBS-dependent activity displays substrate inhibition by excess of NADPH and is strongly stimulated by $NADP^+$ The stimulation is especially pronounced with mercuric reductase, 25-fold under some conditions Neither substrate inhibition nor stimulation by NAD^+ is observed with lipoamide dehydrogenase

Glutathione reductase *Mercuric reductase* *Lipoamide dehydrogenase* *2,4,6-Trinitrobenzenesulfonate* *NAD(P)H oxidase*

1. INTRODUCTION

Glutathione reductase (EC 1.6.4.2), lipoamide dehydrogenase (EC 1.6.4.3), and mercuric reductase are three members of a group of flavoproteins that in addition to FAD also contain a redox active disulfide as part of their active sites [1,2]. These three enzymes have been found to share many spectroscopic, physical and kinetic properties [1,2] and also to possess extensive sequence homology in the active site region [3–9]. In the cell both glutathione reductase and mercuric reductase catalyze oxidation of reduced pyridine nucleotides. The reactions are essentially irreversible at neutral pH. Lipoamide dehydrogenase, on the other hand, catalyzes a reaction that in vitro is freely reversible at neutral pH. In vivo, lipoamide dehydrogenase is part of enzyme systems oxidizing keto acids with concomitant reduction of oxidized pyridine nucleotides. Thus, the redox process proceeds in the opposite direction to that of the processes catalyzed by the other two enzymes.

We have recently shown that glutathione reductase catalyzes the reduction of 2,4,6-trinitrobenzenesulfonate (TNBS) at rates that under optimal conditions amount to at least 20–30% of those of glutathione disulfide (GSSG) reduction under standard assay conditions ([10,11] and manuscript in preparation). Under aerobic conditions the reduced species of TNBS is rapidly reoxidized [11], resulting in an apparent NADPH oxidase activity. This activity was also found to be strongly influenced by $NADP^+$ in a manner that suggests an effector role for the oxidized pyridine nucleotide [11].

We report TNBS-dependent oxidation of reduced pyridine nucleotides catalyzed by glutathione reductase, lipoamide dehydrogenase, and mercuric reductase as well as differences in the effect of oxidized pyridine nucleotides on the different enzymes.

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2. MATERIALS AND METHODS

Glutathione reductase was purified from human erythrocytes essentially as previously described [4]. Lipoamide dehydrogenase (pig) was purchased from Sigma, USA and used without further purification. Mercuric reductase was purified from *Pseudomonas aeruginosa*, strain PAO 9501, carrying the plasmid pVS1 (kindly donated by Dr Simon Silver, Washington University, St. Louis, MO, USA) as described [12]. TNBS was purchased from Sigma. All other chemicals were standard commercial products of high purity. Enzyme activities were measured as the decrease in A_{340} due to the oxidation of NAD(P)H, unless otherwise stated. Hg^0-production was measured as described [13].

3. RESULTS AND DISCUSSION

3.1. *Inhibition by TNBS*

Very low concentrations of TNBS have been found to strongly inhibit GSSG reduction catalyzed by glutathione reductase, presumably by reacting with the active site disulfide; a concentration as low as 50 nM TNBS caused 50% inhibition under standard assay conditions [14]. In contrast, neither lipoamide dehydrogenase nor mercuric reductase were inhibited by 2 μM TNBS. The assay mixture for mercuric reductase contains 1 mM cysteine which could react with TNBS. However, 2 μM TNBS was found to inhibit glutathione reductase by 92% also in the presence of 1 mM cysteine. Even at a concentration of 100 μM, TNBS did not inhibit lipoamide dehydrogenase or mercuric reductase. Under the conditions used for the inhibition studies, 100 μM TNBS does not support any observable NADPH or NADH oxidation (see below), which might otherwise mask an inhibitory effect on lipoamide dehydrogenase and mercuric reductase. In the case of mercuric reductase direct assay based on the production of Hg^0 also demonstrated that 100 μM TNBS did not inhibit the enzyme.

3.2. *Cross-reactivity*

Virtually no cross-reactivity with respect to the natural non-nucleotide substrate can be observed between glutathione reductase, mercuric reductase and lipoamide dehydrogenase (table 1). The specific activity of mercuric reductase is considerably lower than that of the other two, which makes detection of cross-reactivity more difficult. These experiments were performed to verify that the observed TNBS-dependent activities were ascribable to the respective enzymes and not to contamination.

3.3. *TNBS-dependent NADPH oxidation*

Table 2 shows a comparison of the native and the TNBS-supported NAD(P)H oxidations of the three enzymes investigated. Also included is the effect of addition of 0.1 mM $NADP^+$ (NAD^+) on

Table 1

Cross-reactivity between glutathione reductase, lipoamide dehydrogenase and mercuric reductase

Assay	Activity (nmol/min)		
	Glutathione reductase	Lipoamide dehydrogenase	Mercuric reductase
GSSG/NAD(P)H	217	0.9	0
Hg^{2+}/NAD(P)H	0	0.9	9.1
Lipoamide/NAD(P)H	0	112	0

Activities are expressed as the decrease in NAD(P)H concentration in systems containing 0.1 M phosphate (pH 7.6), 1 mM EDTA, 0.1 mM NADPH (glutathione reductase and mercuric reductase) or 0.1 mM NADH (lipoamide dehydrogenase) plus 1 mM GSSG or 0.4 mM lipoamide or 0.1 mM $HgCl_2$ and 1 mM cysteine. The enzyme concentration was 30 nM (FAD) in all cases

Table 2

Comparison of the effect of $NAD(P)^+$ on standard and TNBS-dependent activities

Assay conditions	Activity (nmol/min)		
	Glutathione reductase	Lipoamide dehydrogenase	Mercuric reductase
Standard	217	101	9.1
Standard + $NAD(P)^+$	205	79	8 7
TNBS/NAD(P)H	12	8 6	1.6
TNBS/NAD(P)H + $NAD(P)^+$	29	3.1	17.2

Standard assay conditions were those described in table 1 Lipoamide dehydrogenase was measured with 0.1 mM NADH and 0.1 mM NAD^+ and the other two enzymes with 0.1 mM NADPH and 0.1 mM $NADP^+$. TNBS-dependent activities were measured with 0 5 mM TNBS in the absence of the natural substrate

the activities. The standard activities of glutathione reductase and mercuric reductase are more or less unaffected by the addition of $NADP^+$. If anything, a small inhibition is indicated in the data. In contrast, $NADP^+$ has a marked positive effect on the TNBS-dependent activities, in agreement with what we have reported for glutathione reductase [11]. The effect of 0.1 mM $NADP^+$ is especially pronounced in the case of mercuric reductase, where the TNBS-dependent NADPH oxidation is stimulated more than 10-fold. The activity is raised to a level almost twice that of the standard Hg^{2+} reduction (table 2).

In the case of lipoamide dehydrogenase, on the other hand, both activities, i.e., lipoamide reduction and TNBS-dependent NADH oxidation, are inhibited by 0.1 mM NAD^+ at pH 7.6 (table 2). An activating effect of NAD^+ has been reported and been found to increase with decreasing pH [15,16]. In an experiment run at pH 6.0 the activating effect of NAD^+ on lipoamide reduction was confirmed, but the effect of NAD^+ on the TNBS-dependent NADH oxidation was still negative

Table 3 shows a more detailed study on the effect of $NAD(P)^+$, including effects of varying the NAD(P)H and TNBS concentrations. The TNBS-dependent NADPH oxidation catalyzed by glutathione reductase exhibits strong substrate inhibition by NADPH [11], and the data in table 3 demonstrate substrate inhibition also for mercuric reductase but not for lipoamide dehydrogenase. In addition to the stimulatory effect of $NADP^+$ on the TNBS-dependent NADPH oxidation catalyzed by mercuric reductase, an inhibitory effect of $NADP^+$ at higher concentrations is also indicated. This inhibitory effect can be more clearly seen in fig.1, which at the highest $NADP^+$ concentration, shows that the stimulatory effect decreases with decreasing NADPH concentration.

In conclusion, the experiments with TNBS presented here emphasize the similarities between glutathione reductase, lipoamide dehydrogenase, and mercuric reductase in so far as all three enzymes display high rates of TNBS-dependent oxidation of reduced pyridine nucleotides. On the other hand, the results demonstrate significant differences between the enzymes. Thus, the TNBS-dependent NADH oxidation of lipoamide dehydrogenase is not stimulated by NAD^+, whereas the corresponding oxidase activities of glutathione reductase and mercuric reductase are strongly enhanced by $NADP^+$. Furthermore, glutathione reductase in the standard assay is strongly inhibited by sub-micromolar concentrations of TNBS, whereas the other two enzymes are not. The latter finding suggests that the inhibitory effect of TNBS on glutathione reductase may represent an interaction with the enzyme distinct from that supporting the NADPH oxidation. Consequently, the qualitative and quantitative dif-

Table 3

Effect of pyridine nucleotides on the activities of glutathione reductase, lipoamide dehydrogenase, and mercuric reductase

Conditions			Activity (nmol/min)		
TNBS	NAD(P)H	$NAD(P)^+$	Glutathione reductase	Lipoamide dehydrogenase	Mercuric reductase
0.2	0.02	–	3.6	6.0	1.1
0 2	0.02	0.02	14.3	2.3	8.2
0.2	0.02	0.4	27.0	nd	26.5
0.2	0.1	–	3.6	7.8	0 6
0.2	0.1	0 02	6.9	2.3	2.0
0 2	0.1	0.4	26.1	nd	15.8
1 0	0.02	–	30.0	nd	6.4
1.0	0.02	0.02	86.8	nd	48.0
1.0	0.02	0.4	34.0	nd	52.2
1.0	0.1	–	22.9	nd	4.2
1.0	0.1	0.02	30 8	nd	12.0
1.0	0.1	0.4	72.3	nd	65.9

Conditions were those described in table 1 with TNBS as acceptor substrate at the concentrations indicated. nd, not determined. Lipoamide dehydrogenase was measured with NADH and NAD^+ and the other two enzymes with NADPH and $NADP^+$

ferences between glutathione reductase, lipoamide dehydrogenase, and mercuric reductase with respect to the TNBS-dependent NAD(P)H oxidation and the effects of $NAD(P)^+$ and NAD(P)H on this activity, presented here, indicate that TNBS should be a valuable tool in further elucidation of the differences and similarities between these three enzymes, as well as in gaining more information concerning the catalytic events at the two-electron reduced level.

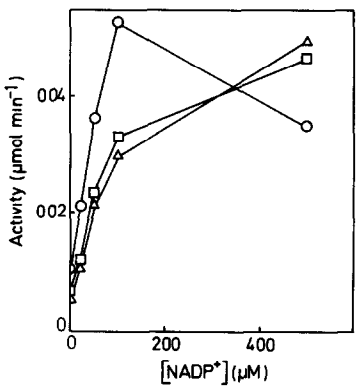

Fig.1. TNBS-dependent NADPH oxidation catalyzed by mercuric reductase. Conditions were those described in table 1. The TNBS concentration was 0.5 mM, and the NADPH concentration was (○) 0.05 mM; (□) 0.08 mM; or (△) 0.1 mM.

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REFERENCES

[1] Williams, C.H. jr (1976) The Enzymes, 3rd edn, 13, 89–173.
[2] Fox, B. and Walsh, C.T. (1982) J. Biol Chem. 257, 2498–2503.
[3] Jones, E.T. and Williams, C H. jr (1975) J. Biol Chem 250, 3779–3784.
[4] Krohne-Ehrich, G., Schirmer, R.H. and Untucht-Grau, R. (1977) Eur. J. Biochem. 80, 65–71.
[5] Matthews, R.G., Arscott, L.D. and Williams, C.H. jr (1974) Biochim. Biophys. Acta 370, 26–38.
[6] Brown, J.P. and Perham, R.N. (1972) FEBS Lett. 26, 221–224.

[7] Burleigh, B D., jr and Wilhams, C H jr (1972) J Biol Chem. 247, 2077–2082.
[8] Fox, B S and Walsh, C T (1983) Biochemistry 22, 4082–4088.
[9] Brown, N.L., Ford, S.J., Pridmore, R.D and Fritzinger, D C (1983) Biochemistry 22, 4089–4095
[10] Carlberg, I. and Mannervik, B. (1982) in· Flavins and Flavoproteins (Massey, V and Williams, C.H jr eds) pp.53–56, Elsevier/North-Holland, New York, Amsterdam, Oxford
[11] Carlberg, I and Mannervik, B (1984) in Flavins and Flavoproteins (Bray, R C et al eds) Walter de Gruyter, Berlin, in press.
[12] Sahlman, L. and Lindskog, S. (1983) Biochem Biophys. Res Commun 117, 231–237.
[13] Schottel, J L (1978) J. Biol Chem 253, 4341–4349
[14] Carlberg, I and Mannervik, B (1979) FEBS Lett 98, 263–266.
[15] Van Muiswinkel-Voetberg, H.V M. and Veeger, C (1973) Eur J. Biochem. 33, 285–291.
[16] Matthews, R G., Wilkinson, K.D , Ballou, D P. and Williams, C H. jr (1976) in: Flavins and Flavoproteins (Singer, T P. ed.) pp 464–472, Elsevier Scientific Publishing Company, Amsterdam.
[17] Thorpe, C and Williams, C.H jr (1976) J Biol Chem 251, 3553–3557
[18] Arscott, L D., Thorpe, C and Williams, C H. jr (1981) Biochemistry 20, 1513–1520