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INTERACTION OF ASCORBATE FREE RADICAL REDUCTASE WITH SULPHHYDRYL REAGENTS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; ascorbate free radical reductase; inactivation studies; sulphhydryl reagents.

Abstract—The involvement of SH-group(s) in ascorbate free radical (AFR) reductase activity was investigated. The incubation of the enzyme with either *N*-ethylmaleimide or mersalyl, or both, resulted in different levels of inhibition. Only inhibition by mersalyl could be partially reversed by thiol-containing compounds, such as cysteine. However, the ability of cysteine to reverse the inhibition was progressively diminished by increasing times of incubation of the enzyme with the inhibitor. Pyridine nucleotides elicited a protecting effect on AFR-reductase when added prior to the inhibitors. This finding was consistent with the presence of a SH-group proximal to the flavin microenvironment of the enzyme.

INTRODUCTION

Ascorbate free radical reductase (AFR-reductase) (EC 1.6.5.4) was found to be essential in maintaining the ascorbic acid system in the reduced state [1]. AFR-reductase catalyses the regeneration of ascorbate from AFR, which is produced by univalent oxidation of ascorbate in both enzymic and non-enzymic reactions [2–5]. Recently AFR-reductase from potato tubers was purified to apparent homogeneity and some kinetic properties of the enzyme were investigated [6]. Thus, a good correspondence between AFR-reductase from potato tubers and that from cucumber [7] was found. Both enzymes were shown to contain SH-group(s) which seem to be involved in their catalytic activity. This paper deals with experiments in which the reactivity of AFR-reductase with the thiol reagents mersalyl (MRS) and *N*-ethylmaleimide (NEM) was investigated.

RESULTS AND DISCUSSION

The kinetics of AFR-reductase inactivation by the sulphhydryl reagents MRS and NEM used in a concentration range of 1–10 μM and 0.1–10 mM respectively are shown in Fig. 1. They differ, as MRS forms a mercaptide from which the free thiol group can be liberated by an added thiol compound, while NEM forms a stable covalent bond and its action is not reversed by added thiol compounds. The inhibition pattern was different. Although two min incubation with 10 μM MRS produced complete inhibition of AFR-reductase activity, inhibition not higher than 50% was found after 15 min incubation with 10 mM NEM. It should be emphasized that neither inhibitor had any effect on the oxidation of ascorbate by ascorbate oxidase under our conditions. These findings indicate that sulphhydryl group(s) of AFR-reductase are involved in the inactivation process.

Further evidence for this hypothesis comes from the fact that thiol containing compounds such as cysteine, 2-mercaptoethanol, and reduced glutathione were able to antagonize the inhibition by MRS, but not the inhibition by NEM, Table 1 shows the effect of cysteine. The thiol alone did not affect AFR-reductase activity. However, inhibition was fully prevented when cysteine was added prior to both inhibitors. Conversely, when cysteine was added after incubating the enzyme with MRS, inhibition was partially reversed, but no removal of NEM inhibition was found. Further, we found that the ability of cysteine to reverse the inhibition by MRS was dependent on the period of incubation of the enzyme with the inhibitor, Fig. 2 shows that such ability decreased with time, being completely lost after 30 min incubation. This finding suggests that the reaction between the inhibitor and the functional SH-group results in the change of AFR-reductase activity by either rendering the SH-group inaccessible to cysteine or by inducing irreversible

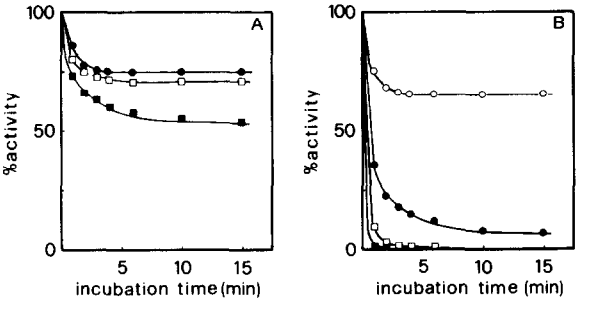

Fig. 1. Kinetics of AFR-reductase inactivation by sulphhydryl reagents. (A) Inactivation by NEM: 0.1 mM (●), 1 mM (□), 10 mM (■). (B) Inactivation by MRS: 1 μM (○), 2.5 μM (●), 5 μM (□), 10 μM (■). The experiments were performed as described in Experimental. 100% activity corresponds to 65 μmol NADH oxidized/min/mg protein.

Table 1. Prevention and reversal of the inhibition by sulphhydryl reagents of the AFR-reductase activity

Experiment	% activity
1 Control	100
2 Control plus 5 mM cysteine	100
3 Inhibition by:	
10 μM MRS	0
2 mM NEM	62
4 Prevention by 5 mM cysteine* of the inhibition by:	
10 μM MRS	100
2 mM NEM	97
5 Reversal by 5 mM cysteine† of the inhibition by:	
10 μM MRS	71
2 mM NEM	60

*Cysteine was added to the reaction mixture just before the inhibitor.

†Cysteine was added after the preincubation of the enzyme with the inhibitor.

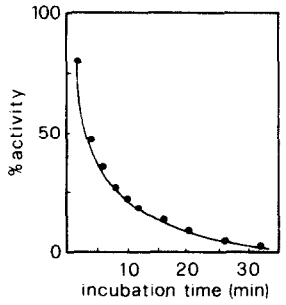

Fig. 2. Effect of the time of incubation of AFR-reductase (7 μg protein) with 10 μM MRS on the ability of 5 mM cysteine to reverse the inhibition of the enzyme activity.

changes of the enzyme structure on removal of the reagent by cysteine.

Preincubation experiments with one or both inhibitors are reported in Table 2. Adding 5 mM cysteine during the course of the reaction did not affect the inactivation of the enzyme induced by preincubation for one min with 2 mM NEM. Conversely, the inactivation induced by preincubating the enzyme for one min with 5 μM MRS was partially reversed by 5 mM cysteine.

When the enzyme was preincubated with NEM for one min prior to adding 5 μM MRS and further incubated for one min, the inhibition was actually comparable to that obtained with NEM alone, and cysteine was without effect. If the order of supplying the two inhibitors was changed, inhibition was comparable to that obtained with MRS alone and was partially reversible by cysteine. However, when the enzyme was preincubated with both inhibitors, a level of inactivation intermediate between those induced by the two inhibitors alone was found. Cysteine led to a reversal of such extent that it could be concluded that only the inactivation by MRS was hindered.

The data of Table 2 indicate that NEM binds to its specific thiol(s) both in the presence and absence of MRS, as shown by the level of activity obtained following cysteine addition. Thus, AFR-reductase reacted with NEM shows less sensitivity to MRS, NEM prevention being almost complete when one min preincubation was allowed.

These findings seem consistent with both inhibitors interacting with the same set of SH-groups. However, the possibility of interaction with different SH-groups cannot be completely excluded.

Other experiments (Table 3) show that the inhibition by sulphhydryl reagents was largely reduced when the enzyme was preincubated with NAD(P)H prior to adding the inhibitor. Conversely, the inhibition was not reduced when NAD(P)H was added after the incubation of the enzyme with the inhibitor. On the other hand, sulphhydryl reagents induced a slight hypochromism, not completely reversible by cysteine, of the two absorption maxima shown by AFR-reductase at 375.5 and 451 nm. These findings are consistent with the presence of an SH-group proximal to the flavin moiety of the enzyme.

Thus, the 'protective' effect of pyridine nucleotides could be due to the formation of a complex E-$FADH_2$-NAD^+ that makes the nearby SH-group inaccessible to the inhibitor.

Table 2. Effect of the preincubation with sulphhydryl reagents on AFR-reductase activity

Experiment		Additions* t = 0	t = 60 sec	t = 120 sec	% activity	% activity with cysteine
Control	ENZ	—	—	R.M.	100	100
1	ENZ	—	NEM	R.M.	59	58
2	ENZ	NEM	—	R.M.	52	51
3	ENZ	—	MRS	R.M.	7	72
4	ENZ	NEM	MRS	R.M.	52	51
5	ENZ	MRS	NEM	R.M.	2	60
6	ENZ	—	NEM + MRS	R.M.	27	58

*At the indicated times 5 μM MRS and 2 mM NEM were added to 7 μg enzyme protein. The reaction mixture (R.M.) was that reported in Experimental. The reaction was started by adding ascorbate oxidase, 5 mM cysteine was added 30 sec later.

Table 3. Effect of NADH on the inhibition by sulphhydryl reagents of AFR-reductase activity

Experiment		Additions* t = 0	t = 30 sec	% activity
Control	ENZ	—	NADH	100
1	ENZ	MRS	NADH	0
2	ENZ	NEM	NADH	62
3	ENZ	NADH	MRS	93
4	ENZ	NADH	NEM	97

*Additions were made at the following concentrations: 10 μM MRS, 5 mM NEM, 0.2 mM NADH. The same protective effect was shown by NADPH.

EXPERIMENTAL

Purified AFR-reductase was prepared as described in ref. [6].

Enzyme assay. AFR-reductase activity was assayed by measuring the oxidation rate of NADH at 340 nm in a reaction mixture containing 0.2 mM NADH, 1 mM ascorbate, 0.1 M Tricine–NaOH buffer pH 8, 7 μg purified enzyme protein, 0.17 U ascorbate oxidase in 3 ml final vol. The reaction was started by adding ascorbate oxidase and the reaction rate was measured within 10 sec. Protein was determined by the method of ref. [8] using bovine serum albumin as a standard.

Inactivation kinetics of the enzyme. The enzyme (70 μg protein) was incubated at 25° with various sulphhydryl reagents in 100 μl of 0.1 M Tricine–NaOH buffer pH 8.10 μl aliquots of incubate were taken at fixed time intervals and immediately assayed as described above.

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