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MODIFICATION OF ARGINYL RESIDUES IN FERREDOXIN-NADP$^+$ REDUCTASE FROM SPINACH LEAVES

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Summary

Reaction of spinach leaves ferredoxin-NADP$^+$ reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.7.1) with α-dicarbonyl compounds results in a biphasic loss of activity. The rapid phase yields modified enzyme with about 30% of the original activity, but no change in the K_m for NADPH. Only partial protection against inactivation is provided by NADP$^+$, NADPH and their analogs, whereas ferredoxin affords complete protection. The reductase inactivated to 30% of original activity shows a loss of about two arginyl residues, whereas only one residue is lost in the NADP$^+$-protected enzyme. The data suggest that the integrity of at least two arginyl residues are requested for maximal activity of ferredoxin-NADP$^+$ reductase: one residue being located near the NADP$^+$-binding site, the other presumably situated in the ferredoxin-binding domain.

Introduction

Previous data on the role of amino acid residues in ferredoxin-NADP$^+$ reductase showed that a sulphydryl group is essential for the activity but is not involved in the binding of the pyridine-nucleotide substrates [1]. More recently, one of us has reported on the presence of a highly reactive lysine located at the NADP$^+$ binding-site of the reductase [2].

Arginine residues have been claimed to participate in the action mechanism of a large number of enzymes, possibly through electrostatic interactions with anionic substrates [3—10]. A simple mechanism in the case of the pyridine nucleotide-dependent dehydrogenases would be the interaction of arginine

residues with the pyrophosphate group of the coenzyme. The hypothesis has some experimental evidence for the NAD^+-dependent enzymes [5,6,11], but not for the $NADP^+$-dependent dehydrogenases. In the latter class, arginine residues have been shown to play a role in the catalytic mechanism [12–15] but only in one case [16] the same residues have been implicated, together with a lysine residue [17], in the binding of $NADP^+$.

For such reason it seemed worthwhile to explore the role of the arginine residues in the coenzyme binding-site of ferredoxin-$NADP^+$ reductase. In this paper we report kinetic studies and chemical modification of the reductase with a class of different α-dicarbonyl compounds as 2,3,-butanedione [4], 1,2-cyclohexanedione [18] and phenylglyoxal [19].

Materials and Methods

Nucleotides, Tris, cytochrome *c* were obtained from Sigma; 2,3-butanedione (Aldrich Chemicals or Merck) was redistilled. 1,2-Cyclohexanedione and phenylglyoxal were also from Aldrich. All other chemicals were analytical grade from Merck. Ferredoxin-$NADP^+$ reductase and ferredoxin were prepared as previously described [20]. Diaphorase activity with ferricyanide as electron acceptor was used to assay the enzyme, unless otherwise stated, as described [2,20].

Modification of the enzyme

1,2-Cyclohexanedione. An 0.3 M solution was freshly prepared in 0.2 M borate buffer (pH 9). The incubation was performed at 30° C in 20 mM borate buffer with 5 μM enzyme.

Phenylglyoxal. A 150 mM phenylglyoxal solution was preparated in 0.1 M phosphate buffer (7.8). 5 μM flavoprotein was treated with phenylglyoxal at 25° C in 0.1 M phosphate buffer (pH 7.8) in the dark.

2,3-Butanedione. Freshly distilled 2,3-butanedione diluted to 1—2% in 50 mM borate buffer (pH 8.5; the pH was accurately readjusted) was used for the inactivation reaction. 80—11 μM enzyme was incubated at 25° C with different concentrations of butanedione.

At various times of the incubation aliquots were withdrawn and assayed for diaphorase activity.

Amino acid analysis. At a given time of the incubation with butanedione, 1-ml aliquots were withdrawn and pipetted in 0.5 ml cold 6 M HCl. The samples were dialyzed for 24 h against 1 M HCl at 4° C, in standard dialysis tubing, transferred to the hydrolysis vials and dried under vacuum [14]. Amino acid analyses were carried out on a Beckman 120 B amino acid autoanalyzer after digestion of the protein in 6 M HCl for 24 h at 106° C, in an evacuated tube.

Fluorescence measurements were made in a Perkin-Elmer Spectrofluorimeter Model MPF2A and adsorption spectra in a Cary Model 118 spectrophotometer.

Results

Kinetics of inactivation by arginine-specific reagents

The reaction of the reductase with monomeric 2,3-butanedione at 25° C

results in a biphasic inactivation curve, with an initial fast phase nearly linear down to 40% of residual activity, followed by a much slower phase which eventually brings about complete inactivation (Fig. 1). Similar inactivation patterns are observed following the activity by the NADPH-ferredoxin reductase reaction.

The kinetics of inactivation has been followed also using 1,2-cyclohexanedione and phenyglyoxal as arginine modifiers (Fig. 2a and b, respectively). A biphasic loss of activity is again observed, although the secondary phase is faster compared to the reaction with butanedione. Thus, the latter compound results the more reactive species and further kinetic studies have been carried out only with this reagent.

Inactivation experiments at different butanedione concentrations (3—100 mM) revealed that the rate of both phases is a function of the reagent concentration, whereas the residual activity at the end of the rapid phase is concentration-independent. According to Ray and Koshland [21], the pseudo-first-order rate constants can be calculated by assuming that the rapid phase yields a partially active enzyme (with 25% residual activity) (Fig. 1, inset). As for the rapid phase, the pseudo-first-order rate constant was calculated by measuring enzyme inactivation as a function of 2,3-butanedione concentration in the range 3—25 mM and found to be a linear function of it. A reciprocal plot of the observed rate constants against butanedione concentration does not yield a finite intercept, this result suggesting a simple bimolecular reaction although an intermediate protein-inhibitor complex with a very high value of K_d can not be excluded. From these data a second-order rate constant of 11.4 $M^{-1} \cdot min^{-1}$ may be calculated. A double logarithmic plot of the half times of inactivation against inhibitor concentration [22] yields the reaction order of 1.04 with respect to inhibitor.

Dialysis against Tris-HCl buffer of the butanedione-treated enzyme (with 15% residual activity), restores about 60—70% of the original activity, whereas no increase is observed after dialysis against borate buffer. These data are fully

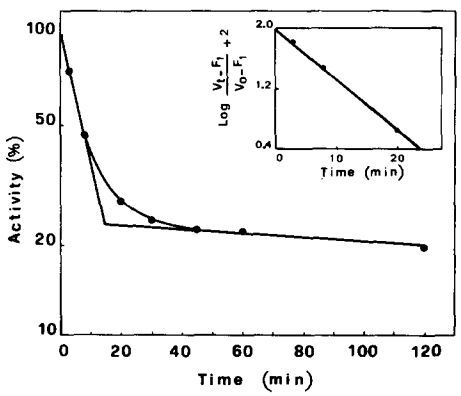


Fig. 1. Inactivation of ferredoxin-$NADP^+$ reductase by butanedione. The incubation was made using 11 mM butanedione. At various times diaphorase activity was measured. Inset: determination of k′ using the value of 24% as F_1, the activity left at the end of the fast phase of the inactivation reaction.

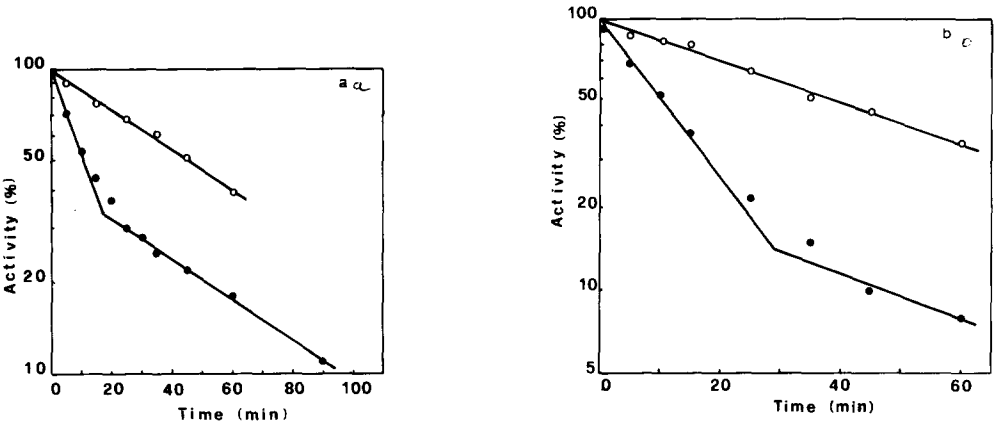


Fig. 2. a, Inactivation of ferredoxin-NADP$^+$ reductase by 1,2-cyclohexanedione. The incubation was performed with 25 mM 1,2-cyclohexanedione in the absence (●———●) and in the presence of 2 mM NADP$^+$ (○———○). b, Inactivation of ferredoxin-NADP$^+$ reductase by phenylglyoxal. The incubation was performed with 100 mM phenylglyoxal in the absence (●———●) and in the presence of 2 mM NADP$^+$ (○———○).

consistent with the arginine being the only modified amino acid residue in our experimental conditions [4,7].

Protection experiments

The effects of various ligands upon the rate of inactivation of ferredoxin-NADP$^+$ reductase by butanedione are illustrated in Fig. 3 and the observed rate constants (k_{obs}) in the case of 10 mM butanedione are reported in Table I.

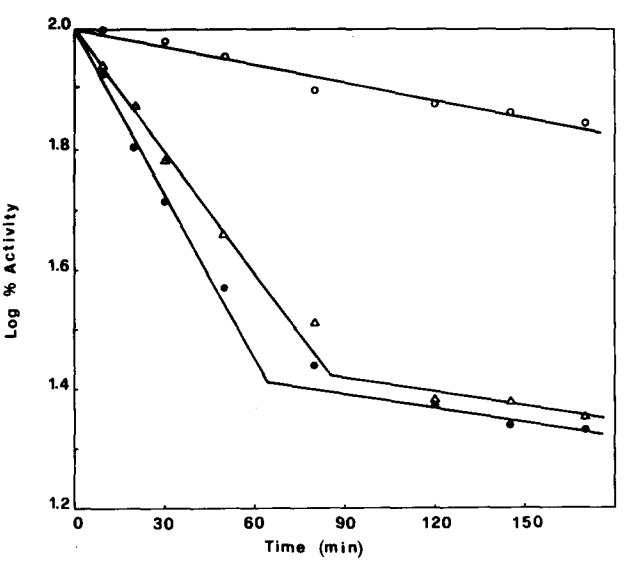


Fig. 3. Effects of ligands on the inactivation pattern of ferredoxin-NADP$^+$ reductase by butanedione. Butanedione concentration was 3.5 mM. No additions (●———●); 2 mM NAD$^+$ (△———△); 2 mM NADP$^+$ (○———○).

TABLE I

RATE OF INACTIVATION OF FERREDOXIN-NADP$^+$ REDUCTASE BY 10 mM 2,3-BUTANEDIONE IN THE PRESENCE OF LIGANDS

Conditions: 50 mM borate buffer (pH 8.5), 9 μM ferredoxin-NADP$^+$ reductase, 10 mM butanedione, 2 mM ligands, 25°C.

Additions	k_{obs} ($\times 10^2 \times min^{-1}$) *
—	6
NADP$^+$	0.57
NADPH	0.70
2′-*P*-ADP-ribose	1.03
2′-AMP	1.25
5′-AMP	3.70
NAD$^+$	4.30

* The k_{obs} were calculated from the slopes of semilogarithmic plots without any subtraction of the slow phase (in accordance to Fig. 5).

NADP$^+$ even at high concentration (2 mM) affords only partial protection; this effect is quite significant in comparison to that produced by NAD$^+$ (Fig. 3). It has been pointed out that borate could form complexes with pyridine nucleotides [13,15], thus causing misleading interpretation on the effects of added nucleotides. The use of phosphate buffer however, does not enhance the protection afforded by 2 mM NADP$^+$. It should be noted that the rate of inactivation in the presence of NADP$^+$ has a value quite similar to that of the slow phase in the absence of the protective agent (Fig. 3). These results have been confirmed using the other two α-dicarbonyl compounds (Fig. 2a and b): in both cases the NADP$^+$ protection is lower than with butanedione and the inactivation curve in the presence of the coenzyme is almost parallel to the slow phase of the inactivation in its absence.

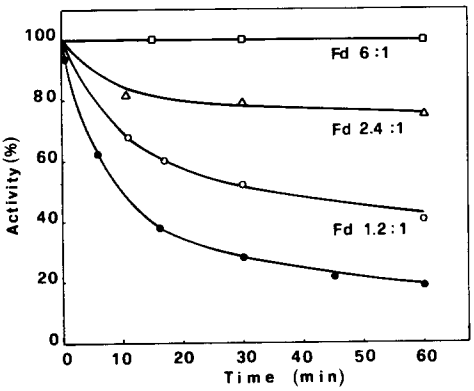
Both the oxidized and the reduced forms of the coenzymes are effective as protective agent. Good protection is given also by 2′-monophospho-5′-diphosphoribose and by 2′-AMP (Table I).

Fig. 4 shows the inactivation patterns in the presence of increasing ferredoxin concentrations. Substantial protection is afforded by an equimolar concentration of ferredoxin and complete protection is probably attained with a 4-fold excess of the protein-substrate.

All these data suggest that the loss of enzyme activity caused by reaction with 2,3-butanedione results from modification in the active-site region.

Modification of arginyl residues

The reaction of 2,3-butanedione with ferredoxin-NADP$^+$ reductase results in a loss of arginyl residues as determined by amino acid analysis. Samples of the butanedione-treated enzyme either in the presence or in the absence of 2 mM NADP$^+$ were analyzed for the amino acid content. The relative time-course of arginine modification is shown in Fig. 5. About 2 arginine residues were modified at the end of the fast phase (20 min, see Fig. 1); one additional arginine residue was lost after prolonged incubation. The presence of NADP$^+$ resulted in a partial protection, the loss of arginine being decreased by approximately one residue.



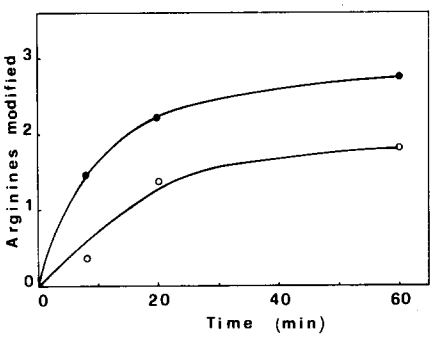


Fig. 4. Effect of ferredoxin on the inactivation reaction of ferredoxin-NADP$^+$ reductase by butanedione. The concentration of butanedione was 3.5 mM. No additions (●———●); 13.5 μM ferredoxin (○———○); 27 μM ferredoxin (△———△); 67.5 μM ferredoxin (□———□). On the figure are indicated the molar ratios of ferredoxin to flavoprotein: 1.2, 2.4 and 6, respectively.

Fig. 5. Arginine modification of ferredoxin-NADP$^+$ reductase with butanedione. 8.39 μM enzyme was treated with 11 mM butanedione, in either the presence (○———○) or absence (●———●) of 2 mM NADP$^+$. Aliquots were removed at the time indicated and processed for amino acid analysis. Results are expressed as the number of modified arginyl residues per mol FAD, based on the loss of arginine as calculated by amino acid analysis. The control enzyme had 8.94 arginine residues per mol FAD.

Properties of partially inactivated enzyme

The flavoprotein at the end of the rapid phase (with about 25—30% residual activity and two arginine residues modified) seems to maintain its native conformation as judged by the spectrum and by protein and flavin fluorescence, which remain unchanged.

Kinetic constants were determined using the enzyme partially inactivated by butanedione or cyclohexanedione (Table II). No significant variation of the K_m for NADPH is observed in comparison to the controls.

TABLE II

KINETIC CONSTANTS FOR MODIFIED FERREDOXIN-NADP$^+$ REDUCTASE

(a) 8.8 μM enzyme was incubated with 11 mM butanedione in 50 mM borate (pH 8.5) at 25°C, then diluted 5-fold with cold borate buffer to stop the inactivation reaction. A control was run under identical conditions. (b) 8.54 μM enzyme was inactivated by 25 mM 1,2-cyclohexanedione in 20 mM borate (pH 9) at 30°C, then diluted 4-fold with cold borate buffer to stop the inactivation reaction. A control was prepared with the same procedure. Kinetic constants were determined as previously described [20].

	Control	Butanedione-treated		Cyclohexanedione-treated	
Activity (%)	100	28	17	44	22
K_m, NADPH (μM)	35	36	38	37	41
K_m, K_3 [Fe(CN)$_6$] (μM)	100	110	—	—	—

Discussion

The ferredoxin-$NADP^+$ reductase from spinach is readily inactivated by a number of α-dicarbonyl compounds. The results with 2,3-butanedione are consistent with three arginine residues out of nine being selectively modified by the reagent; no other amino acid residues are affected. A concomitant modification of the essential lysyl residue [2] can be also excluded on the following grounds: (a) the butanedione adducts formed in our experimental conditions are stable to dialysis against borate buffer, whereas lysine modification should be readily reversible [7]; (b) dansyl chloride which has been shown to modify the active-center lysyl residue [2], is still able to react with the butanedione-treated enzyme and to cause complete inactivation (Zanetti, G., unpublished data).

The biphasic time-course of the enzyme inactivation with all the α-dicarbonyl compounds suggests the presence of at least two classes of reacting arginyl residues. In the fact phase of inactivation by butanedione, a loss of 70% of the original activity is accompanied by the modification of approximately two arginyl residues; a third residue reacts at a much slower rate. A kinetic analysis of the fast phase of butanedione inactivation reveals that loss of activity is correlated to the modification of just one arginine residue. These data indicate the modification of 'non-essential' arginine residues.

The protection afforded by $NADP^+$ to the reductase towards the α-dicarbonyl compounds is highly specific, although not complete. In the same conditions NAD^+, a very poor ligand of the enzyme [23], shows a negligible effect. A likely explanation is based on the kinetic analysis of the inactivation curve. With all the three α-dicarbonyl compounds, the rate of inactivation in the presence of $NADP^+$ is approximately equal to the rate of the slow phase of the inactivation in its absence; thus, the coenzyme seems unable to protect the low-reactive arginine residue whose modification parallels the slow decrease of activity. Ferredoxin in a molar ratio of 6 : 1 to the flavoprotein affords complete protection towards butanedione inactivation. This apparently high value seems quite reasonable considering the negative influence of the ionic strenght on the complex formation [24]. On the other hand, ferredoxin contains a single arginine residue [25]; thus, its addition to the reaction mixture does not alter appreciably the inhibitor-residues ratio. Since ferredoxin partially overlaps the pyridine-nucleotide binding site [26,27] and owing to its molecular size, it is not unreasonable to expect complete protection.

Thus, the integrity of at least two arginine residues seems important for the catalytic function of ferredoxin-$NADP^+$ reductase. The fast-reacting residue, being protected by both $NADP^+$ and ferredoxin, should be located in the active-site region; nevertheless, it does not appear directly involved in coenzyme binding since no significant alteration in the K_m for NADPH has been observed for the partially inactivated enzyme. The slow-reacting arginine residue is protected only by ferredoxin; therefore it may be located in the binding domain of this substrate.

Our results agree with previous reports on other $NADP^+$-dependent dehydrogenases [12—15]. This class of enzymes seems to differ from the NAD^+-dependent dehydrogenases, in which arginine residues play a main role in the binding subsite for the pyrophosphate group of the coenzyme [5,6,11].

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