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A LYSYL RESIDUE AT THE NADP BINDING SITE OF FERREDOXIN-NADP REDUCTASE

GIULIANA ZANETTI

Department of Biochemistry, University of Milano, via Celoria 2, Milano 20133 (Italy)

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Summary

Dansyl chloride, at low molar ratio, inactivates ferredoxin-NADP reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.7.1). The complete protection afforded either by NADP or NADPH suggests a direct involvement of the active site.

Experiments with [*Me*-^{14}C]dansyl chloride showed that about 1.5 residues per flavin were dansylated: by differential labelling experiments using NADP, it has been proved that enzyme inactivation is due to dansylation of one residue. The group modified has been identified as the ϵ-amino group of a lysine. The pH-inactivation profile indicates that this essential group has an apparent pK_a of 8.7.

The dansylated flavoprotein seems to maintain its native conformation; it shows a fluorescent chromophore with a peak at 335 nm. The modified enzyme has lost the capacity to form a complex with NADP, nevertheless it interacts normally with ferredoxin.

It is concluded that the loss of catalytic activity which parallels the dansylation of a lysyl residue occurs because this residue is essential for the binding of the pyridine nucleotide substrate. Protection experiments with a series of coenzyme analogs further indicate that this lysyl residue interacts, most likely, with the 2′-phosphate moiety of NADP(H).

Introduction

The spinach chloroplast ferredoxin-NADP reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.7.1) is an FAD-containing enzyme and thus it belongs both to the pyridine nucleotide dehydrogenases and to the flavoproteins.

Very little is known about the active centre of this enzyme. A sulfhydryl group has been shown to be essential for catalytic activity and to be located near the active site [1]. Any attempt to get a reconstitutable apoprotein has

failed up to now. Our aim was then to identify other functional amino acid residues in the holoenzyme by means of chemical modification.

Lysyl or arginyl residues [2,3] have been suggested as the complementary, positively charged recognition sites for the phosphate moiety of pyridine nucleotides. Reagents suitable for the modification of these residues in protein, under mild conditions, are numerous [4].

Dansyl chloride has been chosen after testing other reagents as pyridoxal phosphate and glyoxal, which were less effective. Dansyl chloride gave rapid inactivation of ferredoxin-NADP reductase at low molar ratio and its inactivation action was completely prevented by the pyridine nucleotide substrates.

A preliminary report of these findings has been presented [5].

Materials and Methods

Pyridine nucleotides, dansyl chloride, dansyl aminoacids, Tris, cytochrome *c*, dithioerythritol were obtained from Sigma Chemical Co.; [*Me*-^{14}C]dansyl chloride was from Sorin, Saluggia (Vercelli). All other chemicals were analytical grade from Merck. Ferredoxin-NADP reductase was prepared as described [6] or with some modifications (Curti and Zanetti, unpublished). Ferredoxin was purified according to the method of Borchert and Wessels [7].

Diaphorase activity with ferricyanide as electron acceptor was used to assay the enzyme, unless otherwise stated. The reaction mixture (3 ml) was kept at 25° C and consisted of 50 mM Tris · HCl (pH 8.2), 0.2 mM NADP, 5 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase in excess and 1 mM ferricyanide. The decrease in absorbance at 420 nm was followed. The NADPH-ferredoxin activity was measured as increase in absorbance at 550 nm with cytochrome *c* as final acceptor, in the above reaction mixture where ferricyanide was replaced by 100 μM cytochrome *c* and 5 μM ferredoxin (the rate-limiting component). Enzyme concentration was determined by absorbance at 458 nm. A molar absorbance of 10 790 $M^{-1} \cdot cm^{-1}$ [8] was used for native and modified protein.

The enzyme was incubated with freshly prepared dansyl chloride in Tris · HCl buffer, since no differences were observed between this buffer and bicarbonate. Acetone (5% final concentration) did not affect the activity even in prolonged incubations at 4° C. The excess radioactive dansyl chloride was removed either by gel filtration on Sephadex G-25 or by acid precipitation. The latter was performed by adding trichloroacetic acid to a 10% final concentration and then 1 mg/ml bovine serum albumin. The samples were centrifuged at 20 000 × *g* for 15 min and washed twice with 2% trichloroacetic acid. This procedure secured full recovery of protein, radioactivity contamination was reduced to 1—2%. Radioactivity was counted using a Packard-Tricarb. The counting medium contained up to 0.2 ml aqueous sample, 0.2—0.5 ml Soluene (Packard) as solubilizing agent and 15 ml scintillation mixture.

Analysis of the dansyl amino acids. The dansylated protein, after acid precipitation and washing with 1 M HCl, was dried and hydrolyzed in 6 M HCl under vacuum for 15 h at 105° C. The dansyl amino acids were separated by thin-layer chromatography on polyamide sheets according to the method of Gray [9].

Fluorescence measurements were made in a Perkin-Elmer Spectrofluorimeter Model MPF 2A and absorption spectra in a Cary Model 118 spectro photometer. The difference spectra were obtained by using the Yankelov cuvettes.

Isoelectrofocusing in polyacrylamide gels was carried out according to the method of Righetti and Drysdale [10].

Results

Reaction of ferredoxin-NADP reductase with dansyl chloride

Ferredoxin-NADP reductase is inactivated rapidly and irreversibly by dansyl chloride, even at low molar ratio (2—3 mol excess reagent per mol FAD yield gives about 70% inhibition). Both the diaphorase and the NADPH-ferredoxin reductase activities are inhibited and with the same time course (Table I); 100% inactivation is eventually attained.

The pH dependence of the inhibition reaction was examined between pH 6.0 and 10.4 at constant ionic strength. Increasing ionic strength inhibits the rate of inactivation. Fig. 1 shows the sigmoidal curve obtained by plotting inactivation against pH. The experimental points at the higher pH values are less well fitted to the theoretical curve, probably because of the enhanced hydrolysis of dansyl chloride by hydroxyl ions [9]. Since dansyl chloride has no dissociable groups in this pH range, one can assume that the rate of the inactivation reaction is dependent on the concentration of the conjugate base form of a functional group on the enzyme. A value of 8.7 for the pK_a of such a group can be calculated from the inflection point of the curve.

Protection against enzyme inactivation

Table II shows the effect of several substrates and analogs on the reaction of ferredoxin-NADP reductase with dansyl chloride.

Both the oxidized and the reduced forms of the coenzymes are effective as protective agents. But whereas NADP and NADPH afford complete protection, NAD and NADH are much less efficient; this reflects possibly the differences in their respective affinities for the enzyme. The good protection given by 2'-AMP seems particularly meaningful.

Determinations of K_d for NADP as protective agent as done by Scrutton and

TABLE I

TIME COURSE OF INACTIVATION OF DIFFERENT ACTIVITIES OF FERREDOXIN-NADP REDUCTASE BY DANSYL CHLORIDE

Incubation conditions: 4°C, 0.1 M Tris (pH 8.8), 3.6 μM enzyme, 40 μM dansyl chloride.

Time of incubation (min)	Residual activity (%)	
	Diaphorase	NADPH-Ferredoxin reductase
14	68	68
25	50	50
47	36	33
74	20	24

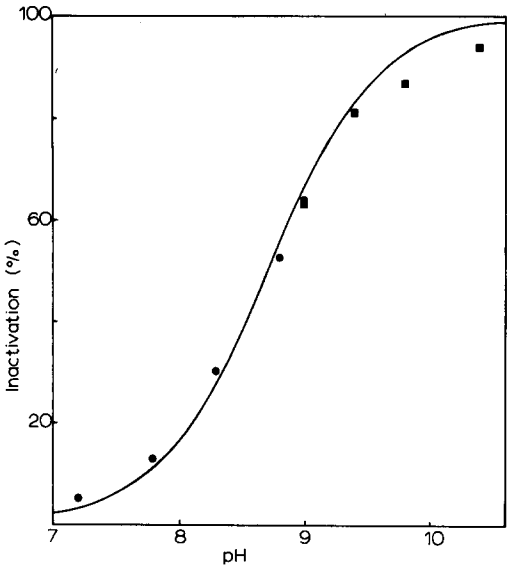

Fig. 1. pH-inactivation profile of ferredoxin-NADP reductase by dansyl chloride. Incubation mixture at 25°C: 9 μM enzyme, 100 μM dansyl chloride, I = 0.1 M final; (●) 0.1 M Tris · HCl, (■) 0.05 M bicarbonate. After 5 min the diaphorase activity was measured. The solid line is the theoretical curve for pK_a = 8.7.

Utter [11], gave values in the range of those published [12], i.e. 30 μM (50 mM Tris pH 8.2) and 80 μM (100 mM Tris pH 8.2). The straight line obtained in this kind of plot [11] passed through the origin, which implies that the flavoprotein-NADP complex can not be inactivated by dansyl chloride.

Stoichiometry of inactivation of ferredoxin-NADP reductase by dansyl chloride

[*Me*-^{14}C]dansyl chloride was used to determine the number of protein residues which were dansylated during the inactivation reaction. About 1.5—1.7 residues per flavin were blocked to reach complete inactivation (Table III). It

TABLE II

PROTECTION OF FERREDOXIN-NADP REDUCTASE AGAINST DANSYL CHLORIDE INACTIVATION BY SUBSTRATES, ITS ANALOGS AND CONSTITUENTS

5.4 μM flavoprotein was incubated in 0.1 M Tris (pH 8.8, 4°C), with 50 μM dansyl chloride, in the presence of 2 mM protective agent for 60 min.

Protective agent	Residual activity (%)
None	26
NADP	98
NADPH	100
NAD	32
NADH	54
Adenosine	25
5'-AMP	32
2'-AMP	51

TABLE III

INCORPORATION OF [*Me*-^{14}C]DANSYL CHLORIDE BY FERREDOXIN-NADP REDUCTASE

Incubation conditions: 0.1 M Tris (pH 8.8, 4°C), 10-fold molar excess dansyl chloride. For gel filtration the enzyme was 36 μM. In the other experiments enzyme concentration varied from 5 to 10 μM. The protein was separated from excess reagent either by Sephadex G-25 filtration or by trichloroacetic acid precipitation as described in Methods.

Treatment	Inactivation (%)	(mol/mol FAD)	
		Dansyl bound per total enzyme	Dansyl bound per inactivated enzyme
G25 filtration	70	1.30	1.85(1.63) *
	65	1.09	1.67(1.46) *
Acid precipitation	73	1.07	1.47
	84	1.20	1.43
	74	1.06	1.43
	60	0.84	1.40

* After acid precipitation.

seems that the higher values obtained after gel filtration were due to a small amount of dansyl chloride not covalently bound to the protein.

Table IV shows the effect of some protective agents on the incorporation of dansyl chloride in the flavoprotein. Actually some radioactivity was found bound to the fully active enzyme which had been completely protected by NADP against inactivation (Tables II and IV). The incorporation was somewhat lower in the presence of NADPH, where the flavoprotein is probably in a reduced form.

The calculated differential values (lines 1, 4 and presumably 5 of Table IV, last column) give the net amount of amino acid residues dansylated to yield inactivation: one residue per mol FAD seems responsible for inactivation.

Further evidence comes from correlation of the residual activity with the labelling of the enzyme in the absence or presence of NADP (Fig. 2). The extra-

TABLE IV

EFFECT OF SUBSTRATES ON THE INCORPORATION OF [*Me*-^{14}C]DANSYL CHLORIDE IN THE FLAVOPROTEIN

Incubation conditions as in Table II. The radioactivity was measured in the protein after acid precipitation.

Addition	(mol/mol FAD)	
	Dansyl bound per total enzyme	Dansyl bound per inactivated enzyme
None	1.08	1.05 *
NADP	0.31	—
NADPH	0.11	—
NAD	0.93	0.91 *
NADH	0.56	0.98 **

* Value calculated by subtracting the incorporation obtained in the presence of NADP

** Value calculated by subtracting the incorporation obtained in the presence of NADPH.

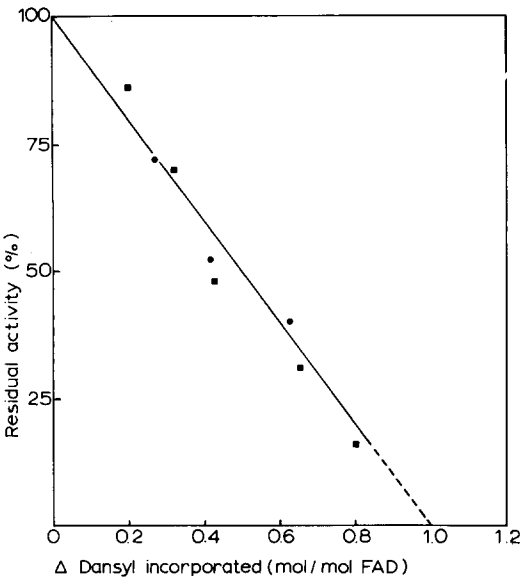

Fig. 2. Correlation of inactivation of ferredoxin-NADP reductase with the differential (± NADP) amount of dansyl chloride incorporated. Incubation conditions: 0.1 M Tris (pH 8.8, 4°C), 10-fold molar excess dansyl chloride, ± 2 mM NADP, enzyme 7.5 μM (■) or 9 μM (●). At various times of incubation, samples of the two mixtures were withdrawn and precipitated with trichloroacetic acid as described in Methods.

polated value comes near to one residue dansylated per molecule of enzyme inactivated.

Identification of the amino acid residue involved in the inactivation reaction

Firstly, it was ascertained that the essential sulfhydryl group [1] was not implicated in the reaction with dansyl chloride. Besides indirect evidence (see Dis-

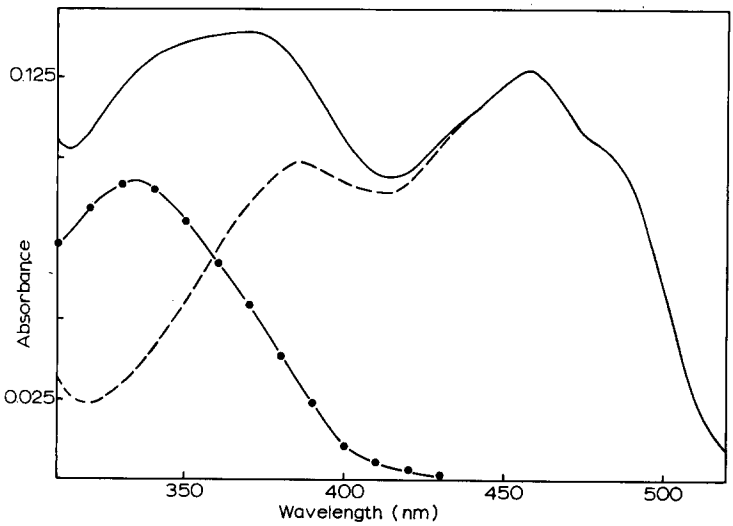

Fig. 3. Absorption spectrum of the modified ferredoxin-NADP reductase. - - - - - -, native enzyme; ———, modified enzyme; ●———●, calculated difference spectrum.

cussion), thiol and imidazol derivatives should be unstable in acid [9]. Dithioerythritol actually did not restore activity to partially inactivated enzyme nor release radioactivity from the protein (5 mM, 2 h at 20° C). Also histidine residues were ruled out since, after treatment with 2 M hydroxylamine [13], all the radioactivity was found bound to the protein.

Analysis by thin-layer chromatography on polyamide sheets [9] of the hydrolyzed flavoprotein, showed a very intense fluorescence spot identifiable as ϵ-dansyl-lysine, together with some dansyl-OH and possibly a small amount of *O*-dansyl-tyrosine. Differences were obtained if dansylation was performed in the presence of the substrates. The amount of ϵ-dansyl-lysine was drastically reduced when NADPH was the protective agent; NADP affected it to a lower extent and NAD not at all.

The identification of ϵ-dansyl-lysine was further substantiated by fluorescence analysis of the digested protein before and after elution from the polyamide sheets. The wavelength maxima for excitation and emission coincided with those of a standard of ϵ-dansyl-lysine in ethanol, i.e. 350 nm and 500 nm respectively. Moreover the calculated difference spectrum (see next section) shows that the flavoprotein which reacted with dansyl chloride gained a new absorbance band centered at 335 nm (ϵ-dansyl-lysine in ethanol has a maximum at 335—340 nm).

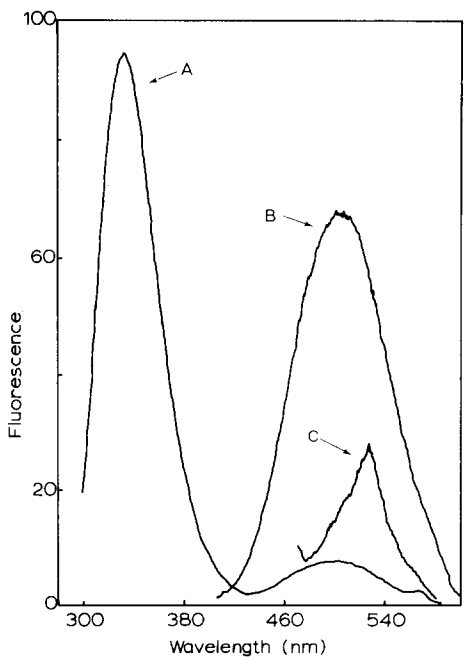

Fig. 4. Fluorescence emission spectra of the modified ferredoxin-NADP reductase. Conditions: 40 mM Tris (pH 8.2, 25° C), 1.5 μM enzyme modified to 70%. (A) excitation 290 nm (slit 4), emission (slit 12), sensitivity 4. (B) excitation 340 nm (slit 4), emission (slit 12), sensitivity 5 (3 times higher than 4). (C) excitation 450 nm (slit 6), emission (slit 12), sensitivity 6 (3 times higher than 5).

Properties of partially inactivated enzyme

The dansylated flavoprotein (70% inactive) seems to maintain its native conformation as judged by protein and flavin fluorescence, which remain unchanged. It shows however, a modification in the absorption spectrum which is ascribable to a chromophore with a peak at 335 nm (Fig. 3). An unusually high value (approx. 6000 $M^{-1} \cdot cm^{-1}$) for the extinction coefficient of the bound dansyl group can be calculated from the incorporated radioactivity (Table III). The environment at the binding site (active center of a flavoprotein) could perhaps be responsible for the high absorption. This group confers to the enzyme a new fluorescence emission band centered around 506 nm (Fig. 4). From the results of the previous section the chromophore should be ϵ-dansyl-lysine. The low value of the emission wavelength maximum would hint at the apolarity of the environment in which the group is sitting.

Further evidence that the protein conformation has been modified only slightly comes from the pattern obtained by isoelectrofocusing. The native flavoprotein presents two main isoenzymes (Zanetti, G., unpublished) as shown

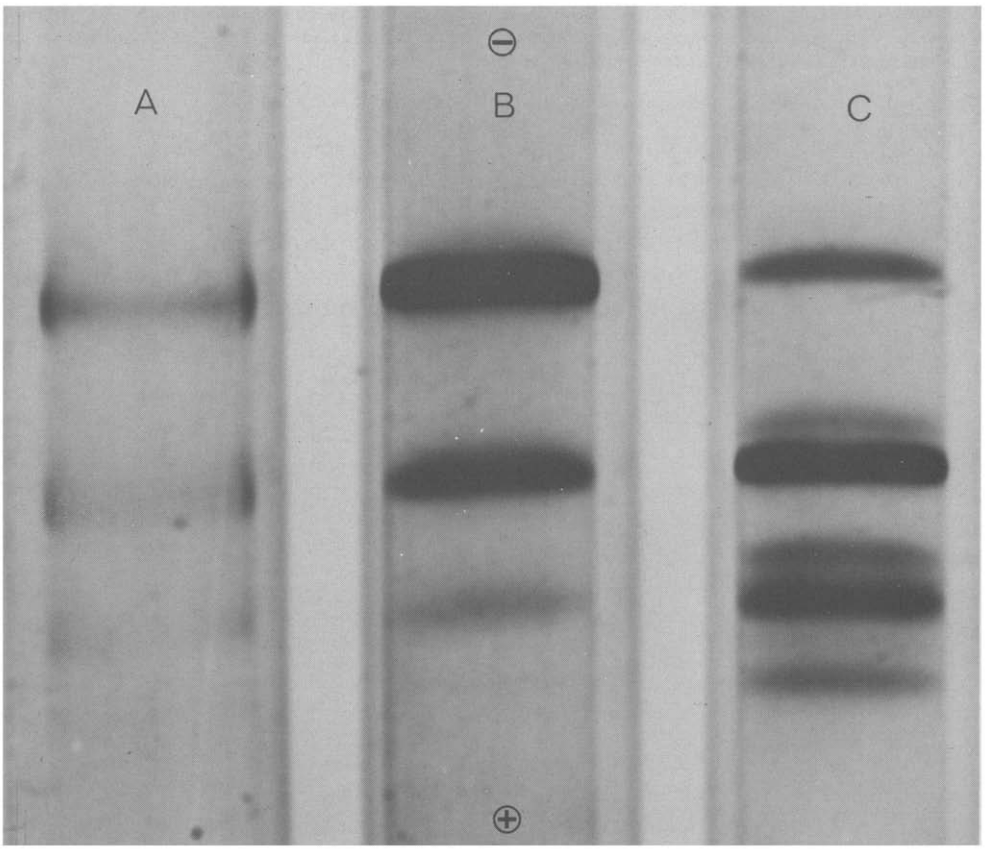

Fig. 5. Isoelectrofocusing pattern of native and modified ferredoxin-NADP reductase. Native and dansylated flavoprotein (70% inactive) were isoelectrofocused in polyacrylamide gels (pH range 4—6) for 4 h. Gel A: native enzyme stained for activity. Gel B: native enzyme stained for protein. Gel C: dansylated enzyme stained for protein.

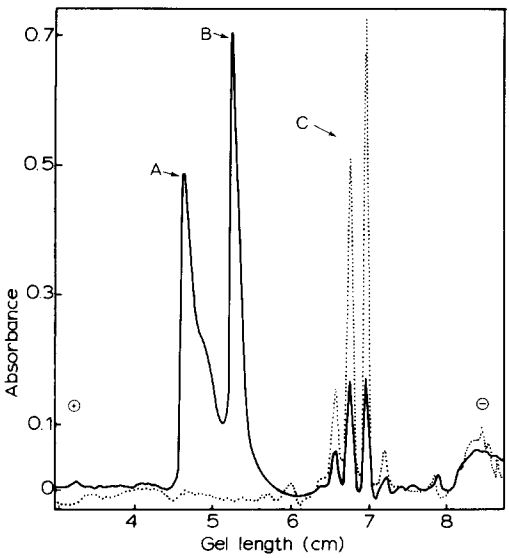

Fig. 6. Interaction of modified ferredoxin-NADP reductase with ferredoxin. The dansylated flavoprotein (92% inactive, after precipitation with $(NH_4)_2SO_4$ and dialysis) was isoelectrofocused with (———) and without (· · · ·) ferredoxin (in excess) in polyacrylamide gels (pH range 4—6). (A) ferredoxin; (B) ferredoxin-flavoprotein complex; (C) dansylated flavoprotein.

in Fig. 5 (A and B). The pattern (Fig. 5, C) of the dansylated enzyme (70% inactive) demonstrates that both isoenzymes bands are displaced by about 70—75% with respect to the anode by only 0.15 units of pH.

The behaviour of the modified flavoprotein with its physiological substrates was investigated. The interaction with ferredoxin was studied by isoelectrofocusing. The native enzyme forms with its protein substrate a complex with an

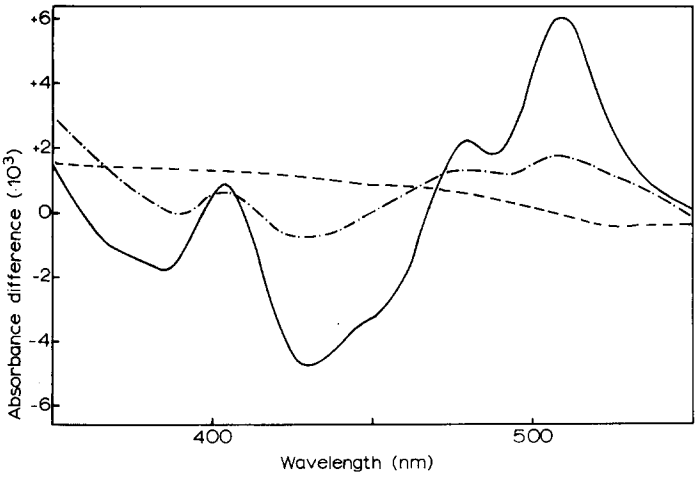

Fig. 7. Interaction of native and modified ferredoxin-NADP reductase with NADP. Curve 1 (———), difference spectrum of native enzyme (21.5 μM in the half-cell) and NADP (1.87 mM). Curve 2 (— · — ·), difference spectrum of dansylated enzyme (21.5 μM, 65% inactive) and NADP (1.87 mM). - - - - - -, baseline.

isoelectric point intermediate between the two proteins and this new band is easily recognizable, at least at short running times (Zanetti, G., unpublished). In Fig. 6 two electrophoretograms are combined to show that about 75% of the dansylated protein forms the complex with ferredoxin; the same percentage is obtained with native enzyme in these conditions. These results suggest that the interaction of the modified flavoprotein (92% inactive) with its protein substrate is not at all impaired.

More pertinent was to ascertain the ability of the dansylated enzyme to interact with the other substrate, i.e. NADP, because of its protective action against inactivation by dansyl chloride. Ferredoxin-NADP reductase is known to show a difference spectrum upon complex formation with NADP [12]. In Fig. 7, the spectra obtained with native and partially inactivated enzyme are compared. All the spectral changes produced in the latter case are accounted for by the amount of active enzyme present in the sample (35%). The dansylated flavoprotein has therefore lost the capacity to bind normally the pyridine nucleotide substrate.

Discussion

In these last years much interest has been devoted to study the participation of specific protein residues to the active center of pyridine nucleotide dehydrogenases, as catalytic species or points of attack for substrates. In this paper strong evidence for a direct involvement of a lysyl residue at the binding site of pyridine nucleotides in ferredoxin-NADP reductase is presented.

The complete protection afforded either by the oxidized or the reduced coenzyme is strongly indicative that the inactivation by dansyl chloride is active site-directed. The conformational changes induced by NADP or NADPH are different (small modifications in the NADP-enzyme complex, dramatic ones when reduction by NADPH occurs [12,14]. Furthermore these coenzymes do not suppress all the radioactivity incorporation, some residues are blocked in their presence without loss of activity. Previously we showed that an essential sulfhydryl group is found near the pyridine nucleotide coenzyme binding site [1]: in that case NADP prevented whereas NADPH accelerated the inactivation; moreover NADP inhibited the action of polar mercurials whereas it favoured that of non-polar species (dansyl chloride is actually a non-polar molecule). Thus a modification remote from or even near to the active site should be regarded as highly improbable.

Since dansyl chloride can interact with several amino acid side-chains [4] it was necessary to identify beyond doubt the residue responsible for inactivation. The essential sulfhydryl group was ruled out on the following grounds: the differences in protection pattern mentioned above; the lack of reactivation and radioactivity release by excess sulfhydryls or acid conditions; the preservation of the native protein conformation. It was excluded either histidine or tyrosine residues, besides the fact that the only amino acid dansylated recovered in meaningful amount by thin-layer chromatography was ϵ-dansyllysine, because the apparent pK_a of 8.7 should be either too high or too low compared to the usual values of these residues in protein, and hydroxylamine did not remove the radioactivity from the enzyme.

It seems important to recall that Keirns and Wang [15], studying the pH dependence of the K_m for NADPH of ferredoxin-NADP reductase, found an ionization with a pK_a = 8.9, which they assigned to the protein moiety. Since it has been reiterated that there is a single binding site for all the pyridine nucleotides [12,16,17] the results obtained with analogs and constituents of NADP (Table II) can be rationalized. Only the adenylic acids, not adenine or adenosine, give protection against dansyl chloride inactivation. 2′-AMP can afford a higher protection than 5′-AMP and even NAD, and to the same degree as NADH. These results, besides showing the obvious dependence on the dissociation constants of these molecules for the flavoprotein, suggest that, most probably, the site of action of dansyl chloride is a lysine residue which interacts with the 2′-phosphate moiety of NADP(H).

Acknowledgements

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