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MOLECULAR HETEROGENEITY OF FERREDOXIN-NADP$^+$ REDUCTASE FROM SPINACH LEAVES

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Summary

Ferredoxin-NADP$^+$ reductase (NADPH : ferredoxin oxidoreductase, EC 1.6.7.1) from spinach leaves has been purified according to a new procedure. The enzyme shows the presence of five molecular forms as identified by isoelectric focusing, namely *a*, *b*, *c*, *d* and *e* with p*I* values of 6.0, 5.5, 5.2, 5.0 and 4.8, respectively. All the bands are catalytically active and are clearly identifiable after the first steps of the purification procedure. The basic pattern of the ferredoxin-NADP$^+$ reductase forms is the same whether extracted from one or many spinach plants and is not affected by the different purification procedures used. Two distinct classes of molecular weight have been found for the isolated forms *b*, *c* and *d* as measured by sodium dodecyl sulphate electrophoresis, with values of 33 000—34 000 for the first and 36 000—38 000 for the latter two forms. Gel electrophoresis in non-denaturing media at different gel concentrations gives the same order of molecular weight values, thus ruling out the possibility that the native enzyme is a dimer, as has been reported by Schneeman, R. and Krogmann, D.W. ((1975) J. Biol. Chem. 250, 4965—4971). No significant kinetic differences were detectable for the isolated forms of ferredoxin-NADP$^+$ reductase.

Introduction

Since the work of Keirns and Wang [1], the presence of multiple molecular forms of ferredoxin-NADP$^+$ reductase (NADPH : ferredoxin oxidoreductase,

Preliminary results of this work have been presented to the 2nd Italian Biochemistry Society Meeting, Venice, 1—4 October 1976.

Abbreviations: PMSF, phenylmethylsulphonylfluoride; INT, 2(*p*-iodophenyl)-3-nitrophenyl-5-phenyltetrazolium chloride.

EC 1.6.7.1) has been reported by several laboratories [2—4], although there are many discrepancies concerning the number of the forms and their physical-chemical properties. Using isoelectric focusing, Keirns and Wang [1] found three forms at pH 6.1, 5.7 and 5.5, with an average molecular weight of 37 000 by sodium dodecyl sulphate electrophoresis. Schneeman and Krogmann [3] reported the presence of two non-identical subunits of ferredoxin-$NADP^+$ reductase with a molecular weight of 45 000—50 000 and 32 000—36 000. Fredricks and Gehl [4] have recently found several forms for crude preparations of ferredoxin-$NADP^+$ reductase, with molecular weights ranging from 50 000 to more than 126 000; the higher molecular weight forms disappear as the purification progresses and the pure enzyme gives essentially two forms at gel electrophoresis with identical molecular weights of 50 000. Considering the above data, it appeared worthwhile to investigate further the ferredoxin-$NADP^+$ reductase from spinach leaves in order to explore the nature and the properties of the multiple forms, and to try to explain the discrepancies reported in the literature. The present paper describes the identification of five different forms of ferredoxin-$NADP^+$ reductase obtained using two different purification procedures, with final isolation of the forms performed by either preparative isoelectric focusing or phosphocellulose chromatography. Some of the five forms have been characterized as to their physico-chemical properties, and the results are discussed in the light of the previous reports.

Materials and Methods

Enzyme purification

Ferredoxin-$NADP^+$ reductase from spinach leaves has been purified either according to a modified procedure of Forti and Sturani [5], or by using an alternative procedure developed in our laboratory:

Step 1. 10 kg of spinach were generally used as starting material. Lots of 250 g of spinach leaves, rinsed in distilled water, were homogenized in a Waring blendor with 300 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM β-mercaptoethanol and 10 μM phenylmethylsulphonylfluoride (PMSF). The collected fractions were filtered through a double layer of cheese-cloth and centrifuged at 11 000 $\times g$ for 45 min.

Step 2. An ammonium sulphate precipitation was carried out, and the fraction precipitated between 35 and 70% of ammonium sulphate saturation was collected. The precipitate was redissolved in a minimum volume of 50 mM potassium phosphate (pH 7.4) and dialyzed against the same medium.

Step 3. Following centrifugation to remove the denatured protein, an acetone fractionation was carried out on the supernatant, taking account of the fact that the maintenance of a low temperature is critical at this stage of purification. During the first 0—45% acetone fractionation, the temperature was kept at 0—2°C. The next 45—75% acetone precipitation was carried out with the temperature of the solution being maintained below —5°C. The solution was then kept at the same temperature for 60 min and centrifuged at 9500 $\times g$ for 50 min. The precipitate was collected, dissolved in a minimal volume of 35 mM Tris buffer (pH 7.4) and dialyzed against the same medium.

Step 4. The dialyzed solution, clarified by centrifugation, was applied to a

DEAE cellulose column (130 × 3.4 cm) previously equilibrated with 35 mM Tris buffer (pH 7.4). The column was then washed with 1.5—2 vols. of the same buffer, after which the molarity of the Tris was increased to 100 mM. The enzyme was eluted in a narrow, sometimes biphasic peak, and the fractions were collected, pooled, and precipitated at 90% ammonium sulphate saturation. The precipitate, after centrifugation, was dissolved in 10 mM Tris buffer (pH 7.4) and dialyzed against the same buffer.

Step 5. The dialyzed solution was applied to a column (40 × 1.9 cm) of a Whatman P 11 phosphocellulose, which had been previously equilibrated with 10 mM Tris buffer (pH 7.4). Following 1.5—2 volumes of washing, the eluant was changed, first to 100 mM potassium phosphate buffer (pH 7.4), and then to 200 mM. All the enzyme active fractions were collected, pooled and concentrated by ammonium sulphate precipitation. Ferredoxin could be purified as a side fraction from the DEAE cellulose column step, according to Buchanan and Arnon [6].

Protein determination

Proteins were determined by the biuret method [7] or by using the ϵ_{275} of $8.5 \cdot 10^4$ [8].

Enzyme assay

Enzyme activity was assayed at 25°C. Cytochrome *c* reductase activity was measured at 550 nm in 1 ml final volume mixture containing 50 nmol of cytochrome *c*, 8.5 nmol of ferredoxin, 200 nmol of NADPH, 80 μmol of Tris buffer (pH 7.8) and enzyme, 80 ng.

A molar extinction coefficient of $19.1 \cdot 10^3$ at 550 nm was used for cytochrome *c* [9]. One unit of activity is defined as that amount which catalyzes the reduction of 1 μmol of cytochrome *c* per min at 25°C.

Diaphorase activity was measured at 420 nm in 1 ml (final volume) mixture containing 0.7 μmol of $K_3Fe(CN)_6$, 0.5 μmol of $NADP^+$, 5.1 μmol of glucose 6-phosphate, glucose-6-phosphate dehydrogenase in large excess, 32 μmol of Tris buffer (pH 7.8) and enzyme, 0.6 μg.

Electrophoresis

Gel electrophoresis was performed according to the method of Ornstein [10] and Davis [11]; sodium dodecyl sulphate electrophoresis was carried out according to the method of Weber and Osborn [12]; isoelectric focusing was performed as described by Righetti and Drysdale [13], either on slabs or on rods using a pH 4—6 gradient. In some cases a preparative isoelectric focusing on gel slab was carried out. Proteins on gel were stained with Coomassie Brilliant Blue R 250, whereas the enzyme activity of the bands was visualized by incubating gel rods or slices in a medium containing 50 mM Tris · HCl buffer (pH 8.2), 0.2 mM NADPH and 0.2 mM INT.

The pH gradient was determined by cutting the gel into pieces, extracting each piece with water and measuring the pH at 2° or 20°C. The isoelectric points of the single ferredoxin-$NADP^+$ reductase forms were determined following identification of the enzyme bands, either directly (by the yellow colour of the flavin coenzyme) or after staining for activity in duplicate experi-

ments. Gel scanning was performed at 625 nm (Coomassie Brilliant Blue) or at 490 nm (INT).

Structural analysis

Carboxymethylation of ferredoxin-$NADP^+$ reductase was carried out according to Harris and Perham [14]; amino acid analysis of the enzyme was carried out on a Beckman 120 B amino acid autoanalyzer equipped with high sensitivity cell, after digestion of the protein in 6 M HCl for 24 h at 106°C in a sealed evacuated tube [15].

NADPH, $NADP^+$, cytochrome *c* (horse heart), glucose-6-phosphate and PMSF were obtained from Sigma. Glucose-6-phosphate dehydrogenase was a product of Boehringer. Ampholine pH 4—6 was supplied by LKB. All the reagents used were analytical grade from Carlo Erba, Italy, unless otherwise stated.

Results

The data concerning the new purification procedure of ferredoxin-$NADP^+$ reductase from spinach leaves are reported in Table I. In this enzyme preparation five different molecular forms can be identified by isoelectric focusing, namely *a*, *b*, *c*, *d* and *e* respectively, going from cathode to anode (Fig. 1a); a similar pattern is observed when ferredoxin-$NADP^+$ reductase is purified according to a modified version of Forti and Sturani [5]. The five ferredoxin-$NADP^+$ reductase forms have p*I* values of 6.0, 5.5, 5.2, 5.0 and 4.8 (measured at 20°C) (Fig. 1b). A more precise determination for form *c*, which is present in greatest amount, gave a value of 5.23 ± 0.06 at 20°C or 5.38 ± 0.06 at 2°C. All of the protein bands are able to reduce INT by direct staining of the gel rods or slabs. After elution from the gel, the enzyme bands show both INT activity and NADPH-ferredoxin reductase activity, the latter being a more physiological and specific test for this flavoprotein. A quantitative scanning of the enzyme as obtained by the pooled fractions after the DEAE cellulose step, gives, on the average, values of 4%, 9%, 60%, 20% and 7% for forms *a*, *b*, *c*, *d*

TABLE I

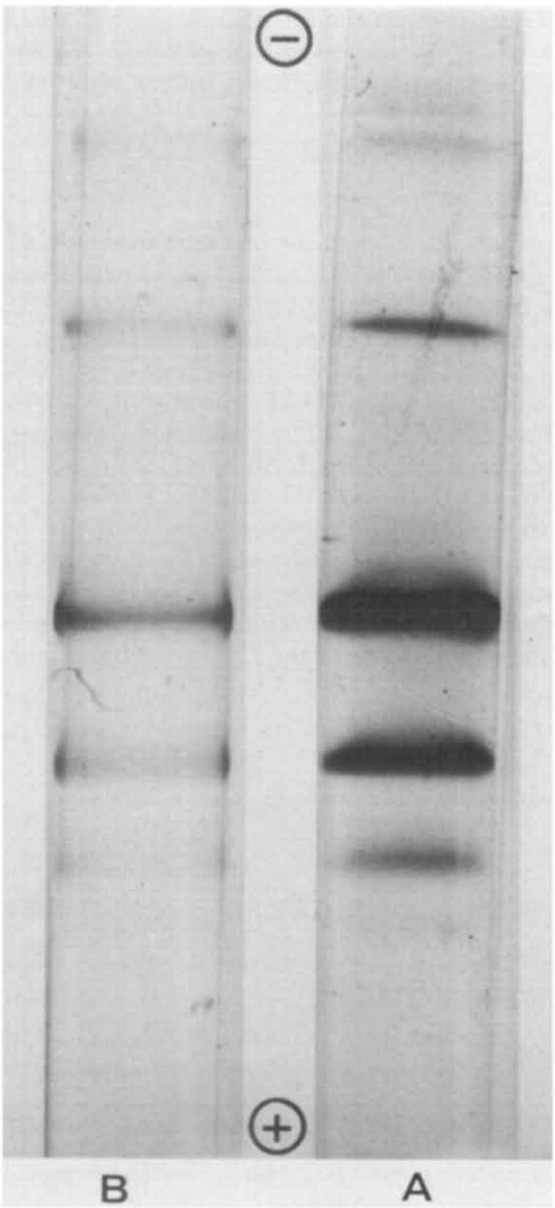
PURIFICATION OF FERREDOXIN-$NADP^+$ REDUCTASE FROM SPINACH LEAVES

Purification steps	Volume (ml)	Total protein (mg)	Total activity units	Specific activity units (mg^{-1}) *	Purification factor	Recovery (%)
Homogenate	8065	80 643	11 892	0.147		100
Ammonium sulphate precipitation	726	32 525	9 595	0.295	2	81
Acetone precipitation	192	4 589	6 957	1.516	10	59
DEAE cellulose chromatography	25.5	75.7	4 901	65.74	447	41
Phosphocellulose chromatography	3.4	26.4	3 608	136.70	930	30

* See under Materials and Methods.

and *e*, respectively. The multiple forms are clearly present at the crude extract stage (see Fig. 2), although at that stage only the forms *c* and *d* are easily identifiable. The amounts of each form are somewhat variable from one preparation to another, and within a single preparation at each stage.

The addition of Triton or 1 mM dithiothreitol during the homogenization did not alter the basic pattern of the multiple molecular forms. Similarly, tests carried out using young or aged leaves from a single spinach plant gave the same results in the isoelectric focusing analysis.



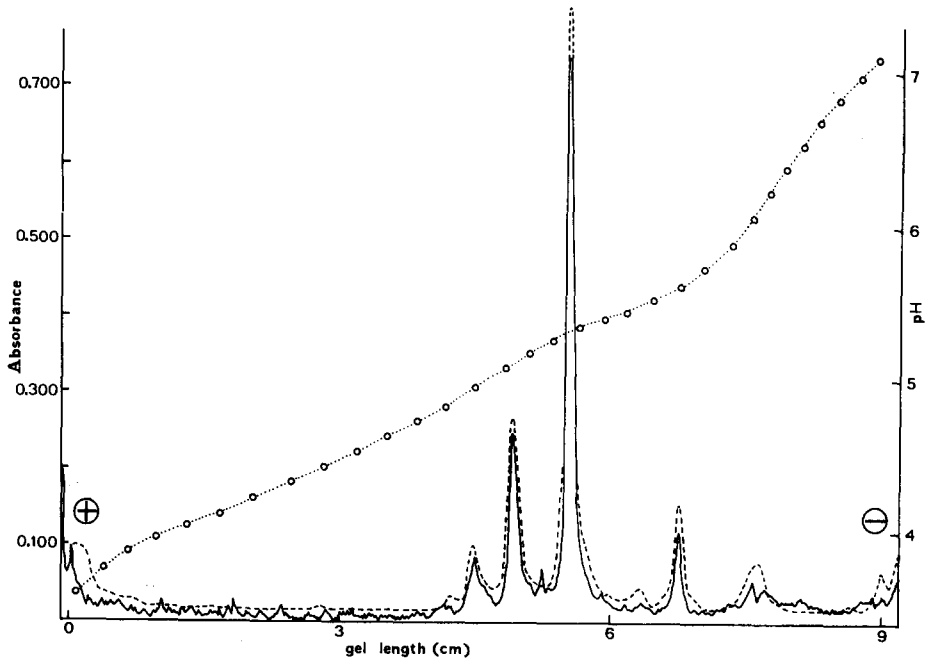


Fig. 1. a, (opposite page) Isoelectric focusing on polyacrylamide gel of the purified unresolved enzyme (pH range 4—6): gel A, stained for protein; gel B, stained for activity. b, (above) Isoelectric focusing pattern of the purified unresolved enzyme. ———, gel stained for protein; - - - - - -, gel stained for activity; ○· · · · · ○, pH gradient profile.

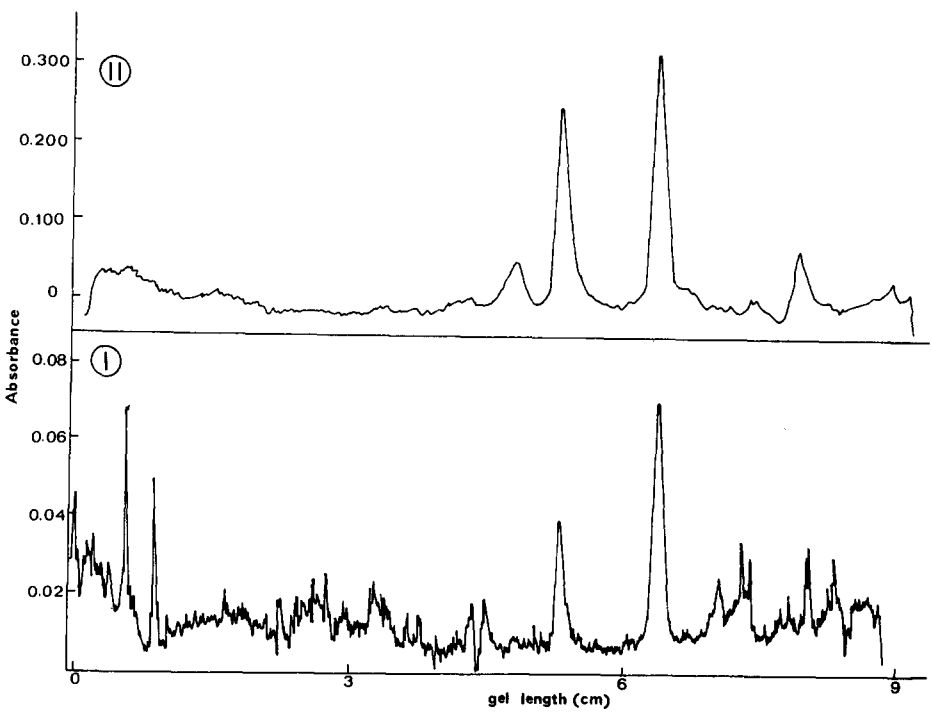


Fig. 2. Isoelectric focusing pattern of ferredoxin-NADP$^+$ reductase at different stages of purification: I, crude extract of the enzyme; II, enzyme after the second purification step. The gels were stained for activity.

Furthermore, treating the unresolved enzyme with 6 M urea and 1 mM dithiothreitol for 60 min at room temperature, and performing the gel isoelectric focusing in 6 M urea, did not modify the bands distribution.

The introduction of the phosphocellulose step in the purification procedure allowed a partial isolation of the multiple forms of ferredoxin-NADP$^+$ reductase, found by isoelectric focusing. Basically the phosphocellulose chromatography, while eliminating protein contaminants which are eluted first, separated the various ferredoxin-NADP$^+$ reductase forms according to the elution profile in Fig. 3. Peak 1 is a mixture of forms *c*, *d* and *e*, with form *c* largely predominant and forms *d* and *e* associated with the leading shoulder. Peak 2 yields essentially form *b*. Peak 3 is mainly composed of ferredoxin-NADP$^+$ reductase form *a*. The latter component is seldom recovered in quantitative amounts because of its low initial amount in the crude extract of spinach leaves. In the modified purification procedure of Forti and Sturani [5] form *d* can be isolated by collecting the tailing fraction from the second DEAE cellulose column and then by performing a further chromatography on phosphocellulose using a linear gradient of 25—125 mM phosphate buffer (pH 7.4). In the same preparation, form *c* was obtained from the main fraction of the DEAE cellulose column followed by phosphocellulose chromatography.

In both purification procedures, complete separation of the various forms of the enzyme could be obtained by a preparative gel-slab isoelectric focusing: in this case a good recovery was achieved with forms *b*, *c* and *d* whereas forms *a* and *e* generally did not give enough material for further studies.

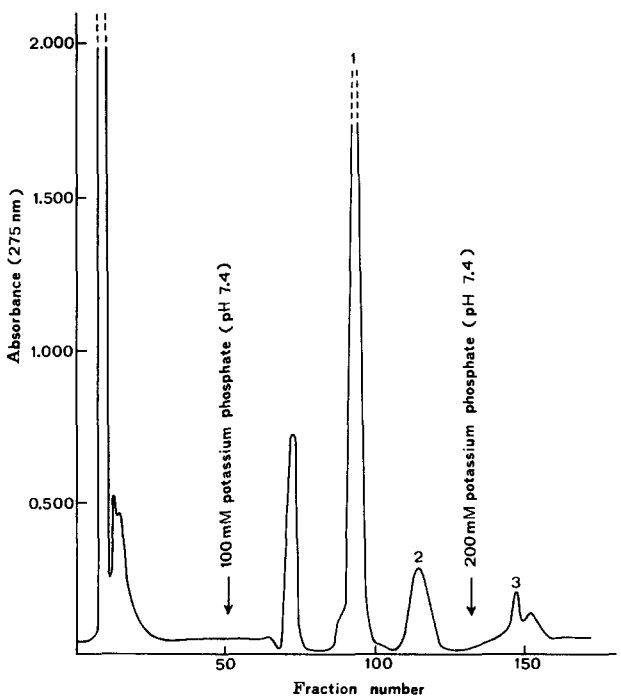


Fig. 3. A typical elution pattern from the phosphocellulose column. Starting buffer: 10 mM Tris · HCl (pH 7.4). At the points shown by the arrows the eluant was changed as indicated.

Kinetic experiments

On the forms *b*, *c* and *d* which appeared homogeneous upon the isoelectric focusing (Fig. 4), a kinetic analysis was carried out and the results are reported in Table II: catalytic activity was tested with $K_3Fe(CN)_6$ as electron acceptor. An activity-pH curve has been also performed on the above ferredoxin-NADP$^+$ reductase forms and the results are reported in Fig. 5. Spectra of the single forms are shown in Fig. 6: all of the three forms have an A_{276}/A_{456} of 8.

Molecular weight analysis

Molecular weight determinations were carried out on the single forms of ferredoxin-NADP$^+$ reductase by employing the gel-multiple concentration method of Hedrick and Smith [16] for the native enzyme and the sodium dodecyl sulphate gel electrophoresis for the single polypeptide chains. By the first method we obtained values of 42 000 for ferredoxin-NADP$^+$ reductase forms *c* and *d* and of 40 000 for form *b*. Sodium dodecyl sulphate gel electrophoresis gave values of 37 000 for forms *c* and *d* and of 34 000 for form *b* (Fig. 7). In one single preparation of the enzyme, a limited amount of form *a* was obtained: sodium dodecyl sulphate gel electrophoresis gave also a value of 34 000 in this case. The summary of molecular weight determinations is shown in Table III.

Amino acid analysis

In order to get further insight into the chemical properties of these ferredoxin-NADP$^+$ reductase multiple forms, an amino acid analysis was carried out

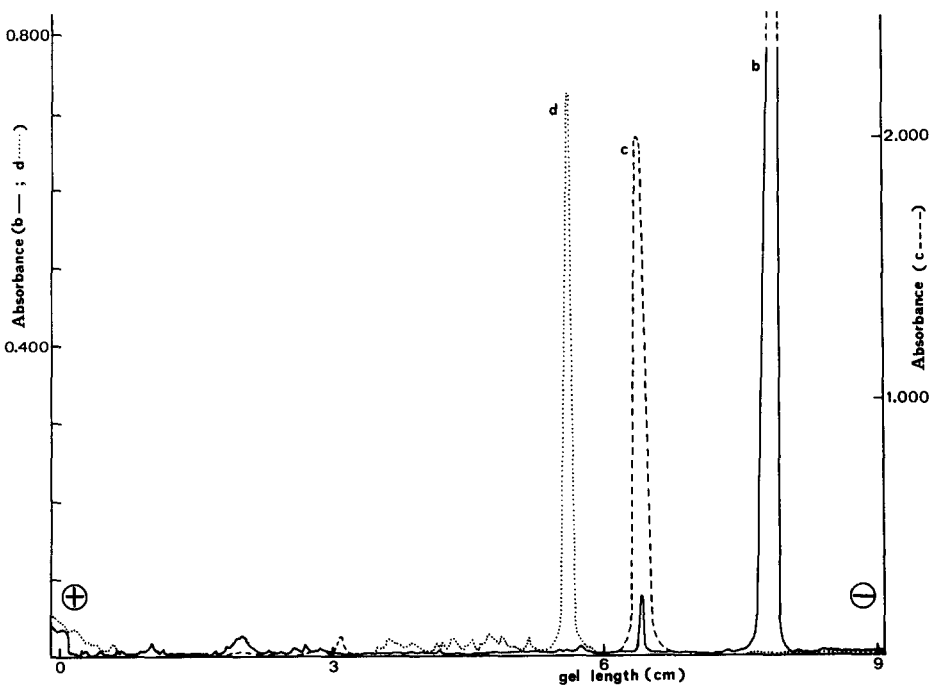


Fig. 4. Isoelectric focusing pattern of the purified ferredoxin-NADP$^+$ reductase forms *b*, *c* and *d*. The gels were stained for proteins.

TABLE II

KINETIC PARAMETERS FOR FERREDOXIN-NADP$^+$ REDUCTASE MULTIPLE FORMS

Conditions: 100 mM Tris · HCl (pH 8.2), 5 mM glucose 6-phosphate and glucose-6-phosphate dehydrogenase in excess, 1 ml final volume.

Ferroxin-NADP$^+$ reductase form	V (eq. min^{-1} · mol^{-1} FAD)	K_m NADPH (μM)	K_m Fe(CN)$_6$ (μM)
b	19 800	36	100
c	30 000	35	97
d	23 000	43	100

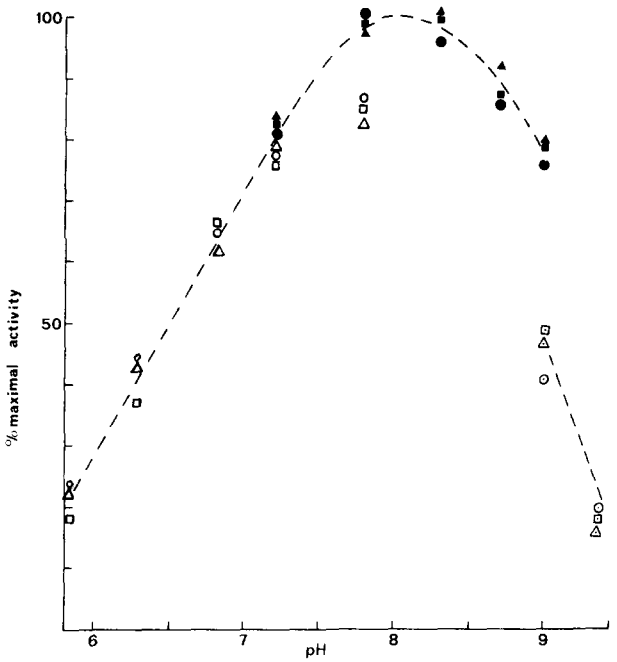


Fig. 5. pH activity curves of ferredoxin-NADP$^+$ reductase forms. The buffers used were: potassium phosphate, pH range 5.8—7.8; ○, form *b*; △, form *c*; □, form *d*, Tris · HCl, pH range 7.2—9.0; ●, form *b*; ▲, form *c*; ■, form *d*, sodium bicarbonate, pH range 9.0—9.4; ⊙, form *b*; ◬, form *c*; ⊡, form *d*, a constant I = 0.15 M was maintained by the addition of NaCl. Assay conditions: 100 μM NADPH, 2.5 μM ferredoxin, 50 μM cytochrome *c*; buffer as above indicated.

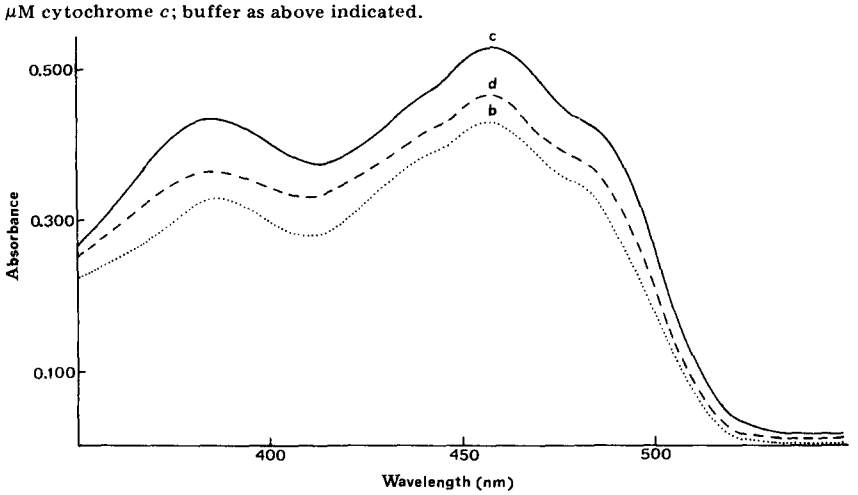


Fig. 6. Absorption spectra of ferredoxin-NADP$^+$ reductase forms *b*, *c* and *d* in 10 mM Tris · HCl (pH 7.4)

TABLE III

MOLECULAR WEIGHTS OF FERREDOXIN-NADP$^+$ REDUCTASE MULTIPLE FORMS

1, molecular weight determined by the gel multiple concentration method [16]; 2, molecular weight determined by sodium dodecyl sulphate gel electrophoresis; 3, molecular weight calculated on the basis of the number of amino acid residues per mol of FAD.

	1	2	3
Form *a*	—	34 000	—
Form *b*	40 000	34 000	33 500
Form *c*	42 000	37 000	35 500
Form *d*	42 000	37 000	—

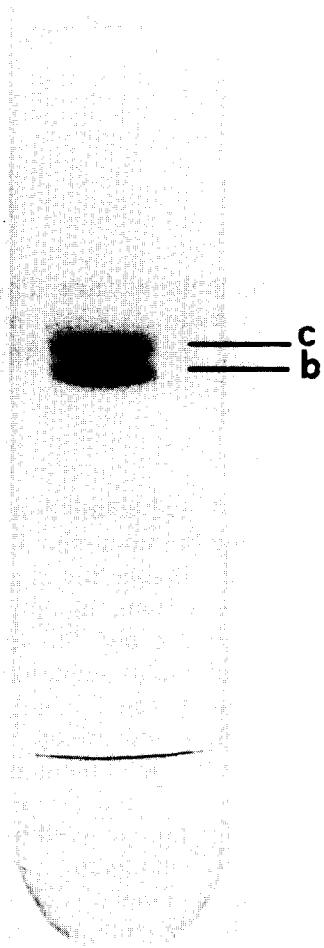


Fig. 7. Sodium dodecyl sulphate gel electrophoresis of ferredoxin-NADP$^+$ reductase, forms *b* and *c*.

TABLE IV

AMINO ACID COMPOSITION OF FERREDOXIN-NADP$^+$ REDUCTASE FORMS *b* AND *c*

Amino acid	Residues per mol of FAD	
	Form *b*	Form *c*
Lysine	34	32
Histidine	5	6
Arginine	9	9
Cysteine [a]	6	6
Aspartic acid	26	29
Threonine [b]	14	16
Serine [b]	17	20
Glutamic acid	32	35
Proline	13	16
Glycine	25	27
Alanine	17	20
Valine	17	19
Methionine	11	11
Isoleucine	13	13
Leucine	21	21
Tyrosine	11	11
Phenylalanine	12	13
Tryptophan [c]	6	6

[a] Determined as carboxymethylcysteine.
[b] Extrapolated values.
[c] Determined according to the method of Liu and Chang [17].

on forms *c* and *b*, which were shown to differ in their molecular weights. The results, expressed as number of amino acid residues per mole of FAD, are reported in Table IV. The molecular weight calculated on the basis of the amino acid analysis were 33 500 and 35 500 for forms *b* and *c*, respectively.

Discussion

The new method for the purification of ferredoxin-NADP$^+$ reductase from spinach leaves reported in the present paper, while shortening considerably the working time requested for the enzyme isolation, does not decrease the classical parameters, viz.: yield, purification factor and specific activity as compared with the previous published methods [1,5,8,18,19]. On the other hand, the introduction of the phosphocellulose step allows a partial characterization of the multiple forms of the enzyme. In this regard, the present results clearly confirm the existence of multiple forms of ferredoxin-NADP$^+$ reductase in spinach leaves. In as much as the previous published data were not in agreement as to number and molecular weights of the enzyme forms concerned [1—4], our results show that at least five forms, namely *a*, *b*, *c*, *d* and *e*, are resolved by isoelectric focusing with p*I* values 6.0, 5.5, 5.2, 5.0 and 4.8 respectively, and we succeeded in isolating forms *b*, *c* and *d* in quantitative yields. The use of PMSF (an inhibitor of the serine dependent proteases) in the initial steps of the enzyme purification excludes the possibility that the various forms arise by proteolytic digestion during the course of the enzyme extraction. The possibil-

ity of artifacts can also be excluded in view of the similar results obtained with enzyme isolated by our purification procedure and by that of Forti and Sturani [5], or by using a single spinach plant as a source rather than pooled spinach leaves. Nor can aging of the starting material account for these five forms considering that old or young leaves gave the same basic pattern of the multiple molecular forms.

Furthermore, isoelectric focusing experiments in 6 M urea + 1 mM dithiothreitol of the unresolved denatured enzyme ruled out the possibility that we are dealing with simple conformers of this flavoprotein.

All the forms are catalytically active and are present at each step of the purification procedure, although the relative amounts of the single enzyme forms vary going from the crude extract to the final phosphocellulose step. A large predominance of form *c* over the other components is generally found at the end of the isolation procedure: possibly the observed enrichment is dependent upon a differential effect of the purification techniques on the various ferredoxin-$NADP^+$ reductase forms. A somewhat different seasonal distribution of the forms was also observed, as well as a variant pattern, after screening of several varieties of spinach leaves (Gozzer, C., and Galliano, M., unpublished).

A kinetic study on the ferredoxin-$NADP^+$ reductase forms *b*, *c* and *d* does not at the present time show significant differences when $K_3Fe(CN)_6$ is used as electron acceptor; nor does the pH profile curve reveal any differences between the three forms. On the other hand it is well known that ferredoxin forms a stable complex with ferredoxin-$NADP^+$ reductase [20] which can be identified by isoelectric focusing [21]. When this method was applied to our forms *b*, *c* and *d*, the same amount of bound enzyme resulted when titration was carried out with limiting amounts of ferredoxin. We can not exclude the possibility that more thorough investigations may, in the future, reveal some significant catalytic divergencies among the various ferredoxin-$NADP^+$ reductase forms. In this regard it may be noted that Margoliash et al. [22], using cytochrome *c* of various species, found large differences in cytochrome oxidase activity only after experiments were conducted at low ionic strength.

As for the molecular weight of these ferredoxin-$NADP^+$ reductase forms, our present results are not in agreement with the values reported by Schneeman and Krogmann [3] or by Fredricks and Gehl [4]. Under our experimental conditions we obtained molecular weight values in the range of 34 000—42 000, the upper limit being the value found for ferredoxin-$NADP^+$ reductase form *c* with the gel method of Hedrick and Smith [16], the sodium dodecyl sulphate electrophoresis and the analysis of the amino acid residues per mol of FAD giving values of 36 000—37 000 for the same form. The latter two methods (see Table III and Fig. 7) tend to demonstrate the existence of two distinct molecular weight classes of ferredoxin-$NADP^+$ reductase forms, with values of 33 000—34 000 and 36 000—38 000, respectively. We did not observe, as reported by Schneeman and Krogmann [3], any dimeric form of the enzyme of molecular weight 85 000 composed of two non-identical subunits of molecular weight 45 000—50 000 and 32 000—36 000, respectively. At the gel filtration on Sephadex G 150 our form *c* gave a molecular weight of 42 000, thus confirming the data reported in Table III. It seems conceivable that the single band observed by the above authors with disc gel electrophoresis may be a mixture

of our multiple molecular forms: obviously, after extraction, this band will split at least into two bands upon sodium dodecyl sulphate electrophoresis.

Finally, as for the origin of those multiple molecular forms, we have at the present time only tentative answers. The amino acid composition analysis of the forms so far examined do not provide sufficient evidence for homologies or divergencies between the two molecules, considering also that we do not know the amide contents of the two forms. A possible multiple gene locus origin cannot be excluded, although multiple alleles at a single locus seems more likely because of the fact that polyploidism is a widespread phenomenon among the spinach plants. Nor can the hypothesis of a post-transcriptional modification of the enzyme primary structure be ruled out on the basis of the present results.

Acknowledgments

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