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Immunochemical Characterization of Ferredoxin from *Halobacterium* of the Dead Sea

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An immunological approach, based on both antigen binding tests and radioimmunoassay, has been used for the comparative study of ferredoxin from *Halobacterium* of the Dead Sea and ferredoxins from a variety of sources. The halophilic ferredoxin was not cross-reactive with the plant, and the mammalian ferredoxins tested. On the other hand, it exhibited up to 80% of antigenic cross-reactivity with a ferredoxin from another extreme halophilic organism, *Halobacterium halobium*. It is therefore proposed that these two ferredoxins represent a class of halophilic 2-Fe ferredoxins, distinct from the plant and algal classes.

The halophilic apo-ferredoxin in its reduced and alkylated form is antigenically indistinguishable from the holo-protein, whereas the non-reduced form of the apo-ferredoxin reacts better than the holo-protein with antibodies towards the latter. This phenomenon is attributed to the fact that upon removal of the prosthetic group, the apo-ferredoxin becomes aggregated and thus its antigenic valence is increased. Competition experiments indicated that the apo-ferredoxin contains all the antigenic determinants present on the holo-protein and that the prosthetic group apparently does not play any direct role in the antigenic specificity of the ferredoxin molecule.

Ferredoxins are iron-sulfur electron-transfer proteins, which are versatile from both structural and functional points of view [1–3]. They take part in a broad range of bioenergetic reactions in bacteria, algae, higher plants and animals. Though different ferredoxins share may properties, striking structural differences exist between the various classes of these iron-sulfur proteins [4]. Thus, for example, ferredoxins containing 2,4 and 8 Fe-S centers ranging in molecular weight from 6000 to 30000 are found in various sources. Initially, 4-Fe and 8-Fe ferredoxins were termed bacterial-type ferredoxins, in contradistinction with the plant-type ferredoxins, which contain only 2 iron atoms per molecule. However, some ferredoxins isolated from non-plant sources, such as the aerobic bacteria *Pseudomonas putida* [5], *Azotobacter* [6] and *Escherichia coli* [7], as well as from mammalian adrenal gland [8] were also found to be 2-Fe ferredoxins.

Recently, 2-Fe ferredoxins were also isolated from the extremely halophilic bacteria *Halobacterium halobium* [9] and *Halobacterium* of the Dead Sea [10] and their structural and redox properties studied. Although these ferredoxins were found to share many features with the plant-type ferredoxins, differences between these two groups were also noted, and it was, therefore, of interest to study the relationships between these classes by a comparative immunochemical investigation.

The use of immunochemical methodology as a probe for protein structure, has contributed a great deal to comparative studies of various protein antigens including structural proteins, enzymes, hormones, etc. [11]. In some protein antigens this approach has shed light on the structure-function relationships in the macromolecules under study [12].

One of the main contributions of immunochemical methodology to protein chemistry was made in the field of molecular evolution. It was found that, for a variety of proteins, including some structurally well defined proteins such as cytochrome *c* [13–15], lysozyme [16,17], trypsin [18] and many others [12, 19], the phylogenetic distance expressed by the degree of similarity in both primary and tertiary structure is usually reflected in the extent of antigenic cross-reactivity between the related proteins.

The use of immunological reagents proved to be highly informative in the study of the relationships

Abbreviations. H-ferredoxin, ferredoxin of *Halobacterium* of the Dead Sea; NaCl/P_i, phosphate-buffered saline.

between some proteins, which contain prosthetic groups and their apo-derivatives. Thus, in some proteins, such as myoglobin, a difference is known to exist both in conformation [20] and in antigenic specificity [21,22] between the holoprotein and the apoprotein. However, in many other examples the holoproteins and the apoproteins were found to be antigenically indistinguishable [19,23,24].

As for the immunochemical properties of ferredoxins, extensive studies have been performed on the well characterized 4-Fe ferredoxin from *Clostridium pasteurianum* [25,26]. However, only limited information exists with regard to the antigenic properties of the 2-Fe ferredoxins. Tel-Or *et al.* [27] have recently surveyed the antigenic properties of a variety of plant-type, algal as well as 4-Fe and 8-Fe bacterial ferredoxins. They have shown that different degrees of cross-reactivity do exist between the various ferredoxins of each of the two former groups, whereas antibodies to bacterial-type ferredoxins reacted only with the homologous proteins used for immunization.

In the present study the antigenic properties of ferredoxin from *Halobacterium* of the Dead Sea were compared to those of ferredoxins of a variety of species, as well as to these of the apoferredoxin obtained by removal of the iron-sulfur complex.

MATERIALS AND METHODS

Ferredoxins

Ferredoxin from *Halobacterium* of the Dead Sea (H-ferredoxin) and from *Halobacterium halobium* were prepared according to the procedure described for ferredoxins from *Halobacterium* species [10]. Both had a ratio of absorbance $A_{420}:A_{280}$ of 0.32, Ferredoxins from other sources were gifts as follows: *Beta vulgaris* (Swiss chard) ferredoxin from Dr M. Schönfeld (Botany Department, Tel-Aviv University, Ramat Aviv, Israel) and from Miles-Yeda (Rehovot, Israel); *Spirulina maxima* ferredoxin from Drs K. K. Rao, R. Cammack and D. O. Hall (Department of Plant Sciences, University of London, King's College, U. K.); adrenodoxin from Dr T. Kimura (Department of Chemistry, Wayne State University, Detroit, Michigan, U.S.A.); and lettuce ferredoxin from Dr Avron's group (Department of Biochemistry, The Weizmann Institute of Science, Rehovot, Israel). All ferredoxins were found to be pure in gel electrophoresis in sodium dodecyl sulfate-polyacrylamide gels.

Preparation of Apo-H-ferredoxin

A solution of ferredoxin of *Halobacterium* of the Dead Sea (15.4 mg in 0.7 ml of 4.3 M NaCl) was acidified with 0.1 ml of 2.5 M HCl. This treatment resulted in precipitation of the protein, which after 1 h at 25 °C was spun down ($12000 \times g$, 20 min), and washed with an acidic solution of 4.3 M NaCl. The washed precipitate was suspended in a minimal volume of water, neutralized with ammonia and lyophilized. Reduced and alkylated apo-H ferredoxin was prepared by incubating apo-ferredoxin at pH 7.5 with 10 mM dithiothreitol for 1 h at 37 °C, and subsequently at pH 8.2 with 2.5-fold molar excess of iodoacetamide (B.D.H., U. K., recrystalized from hot benzene).

Analytical Gel Filtration

Gel filtration on Sephadex G-100 was performed in glass-bead-filled column [28] (1.6×85 cm). The protein solution (1 ml) was loaded on top of the gel and fractions of 2.2 ml were collected. The migration of the different proteins was followed either by monitoring the absorbance at 420 nm or by determining the radioactivity, in cases where radioactively labelled antigen was used.

Photo-reduction of Ferredoxin by Chloroplasts

H-ferredoxin solution (1.8 mg/ml) was preincubated for 15 min at 37 °C with different amounts of antiserum to H-ferredoxin. It had previously been established that H-ferredoxin is photoreducible (M. M. Werber, unpublished experiments). The reduction was performed by illuminating the sample containing the treated ferredoxin at a final concentration of 120 µg/ml and lettuce chloroplasts (70 µg chlorophyll) with light of 600 – 720 nm. The process of reduction was followed by monitoring the difference in absorbance between 497 and 540 nm in an Amino-Chance dual wavelength spectrophotometer [29].

Chemical Reduction of Ferredoxin

Ferredoxin (1.8 mg/ml) of either *Halobacterium* of the Dead Sea or *B. vulgaris* was preincubated for 15 min at 37 °C with excess or without antiserum to H-ferredoxin or to *B. vulgaris* ferredoxin. The reduction was performed in a Thunberg tube under anaerobic conditions by the addition of solid sodium dithionite to a buffered 50 mM (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 6.9) solution of the ferredoxin (0.9 mg/ml) and the antisera. The extent of reduction was followed by monitoring the changes in absorbance in the range 420 – 600 nm.

Coupling of Ferredoxin to Multi-Chain Polyalanine

H-ferredoxin and its apo-protein as well as *B. vulgaris* ferredoxin were coupled to multi-chain poly-

(DL-alanyl)-poly(L-lysine) [30] using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (The Ott Chemicals Co., U.S.A.) as a coupling reagent. Ferredoxin (10 mg) was mixed with poly(L-alanyl)-poly(L-lysine) (35 mg) and the carbodiimide (10 mg) in 4 ml of phosphate-buffered saline ($NaCl/P_i$) and incubated for 24 h at 25 °C. Material that precipitated during the coupling procedure (less than 5%) was removed by centrifugation and the solution was exhaustively dialyzed against $NaCl/P_i$. The sedimentation velocity pattern of the conjugates, obtained in a Beckman analytical ultracentrifuge (model E), equipped with a spectrophotometric scanner, indicated that all the ferredoxins underwent complete coupling to the poly(DL-alanyl)-poly(L-lysine) backbone.

Antisera

Antisera towards H-ferredoxin, *B. vulgaris* ferredoxin and apo-H-ferredoxin were evoked in rabbits by three injections at 10 days intervals, each of 1 mg of the respective (poly(DL-alanyl)-poly(L-lysine) coupled ferredoxin emulsified in complete Freund's adjuvant (Difco, U.S.A.). Sera were collected 10 days after the last injection.

Iodination of Ferredoxins

20 µg of ferredoxin of the various sources (as will be specified) were labelled with ^{125}I by the lactoperoxidase method according to Marchalonis [31]. The labelled protein was separated from unbound iodide by gel filtration through a Sephadex G-25 column equilibrated with $NaCl/P_i$ containing 1 mg/ml gelatin. Small aliquots were frozen and kept at −20 °C until used. The specific activity of the radioactive ferredoxins was usually about 30 mCi/mg protein. It was ascertained that the exposure to H_2O_2 during the reaction with lactoperoxidase did not affect the visible spectrum of ferredoxin.

Antigen Binding Assay

Titration of antisera in respect to their binding capacity for radioiodinated ferredoxin was performed as follows; serial dilutions of the respective antiserum in non-immune goat serum, in a volume of 100 µl, were pipetted into micro test-tubes (Eppendorff, W., Germany), followed by 50 µl of $NaCl/P_i$ containing 1 mg/ml gelatin. The radioactive antigen (about 40000 counts/min in 50 µl), diluted in $NaCl/P_i$-gelatin was then added and the mixture was mixed, incubated for 2 h at room temperature and subsequently for 1 h at 4 °C. The immunoglobulin fraction was then precipitated by the addition of 600 µl of 60% saturated ammonium sulfate. After at least 3 h at 4 °C, the precipitate was spun down in an Eppendorf 3200 centrifuge and 600 µl of the clear supernatant were aliquoted and the radioactivity determined. For low values of binding the measurement was verified by counting the radioactivity in the washed precipitate. Non-specific precipitation did not exceed 5% and up to almost 100% of the protein could be bound under conditions of antibody excess.

Radioimmunoassay

Competition experiments were conducted for the determination of the concentration or the antigenic properties of the various unlabelled ferredoxins. The experiment was performed as described for the antigen binding assay, except that 50 µl of solution containing the tested ferredoxin were added instead of the $NaCl/P_i$-gelatin and mixed with the labelled ferredoxin prior to the addition of 100 µl of the antiserum. The concentration of the antiserum was kept constant, a 1:100 dilution in non-immune goat serum.

Determination of Ferredoxin Concentration

The molar concentrations of the ferredoxins from various species were determined from their visible absorption, using a value of $\varepsilon = 9600\ M^{-1}\ cm^{-1}$ at the peak around 420 nm. The concentrations were converted in units of mg/ml, using the published values [4] for the molecular weights of the various ferredoxins. In the case of the H-ferredoxin and H-apoferredoxin we made use of our recently reported data, *i.e.* 0.61 per mg/ml at 420 nm and 1.47 per mg/ml at 280 nm for the holo-ferredoxin and apo-ferredoxin respectively [10].

RESULTS

Antigenic Relationships between Ferredoxin of Halobacterium *of the Dead Sea and Various 2-Fe Ferredoxins*

Antisera towards ferredoxins of either *Halobacterium* of the Dead Sea or *B. vulgaris* were tested for their capability to bind radioiodinated ferredoxins of the two sources. Several ten-fold dilutions of the tested antisera were mixed with the ^{125}I-labelled ferredoxin (40000 counts/min) and subsequently precipitated with ammonium sulfate as described under Materials and Methods. The results, depicted in Fig. 1 indicate that each antiserum was capable of binding the homologous antigen to an extent of over 95%. Elevating the serum concentrations from $1:10^4$ to $1:10^2$ dilutions resulted in a proportional increase in the binding of the radioactive ferredoxin. However, the two antisera, even at the highest concentrations (1:10 dilution) were completely incapable of binding the heterologous

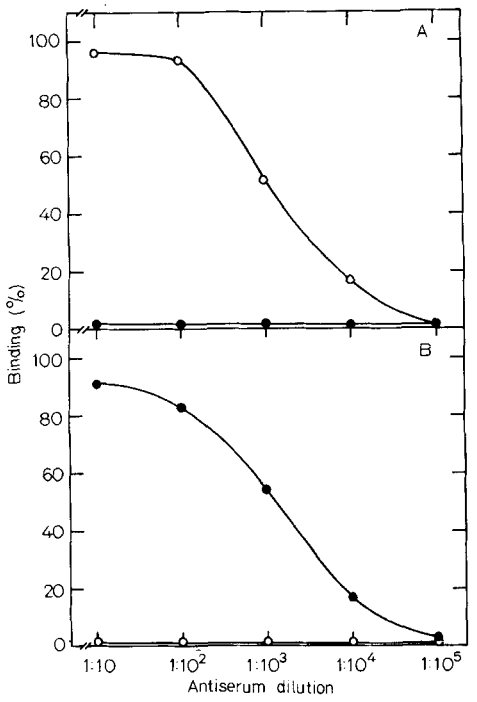

Fig. 1. *Antigen binding tests of H-ferredoxin (○) and* B. vulgaris *ferredoxin (●) with antiserum towards H-ferredoxin (A) or towards* B. vulgaris *ferredoxin (B).* Non-specific binding (< 5%) was subtracted from the experimental values

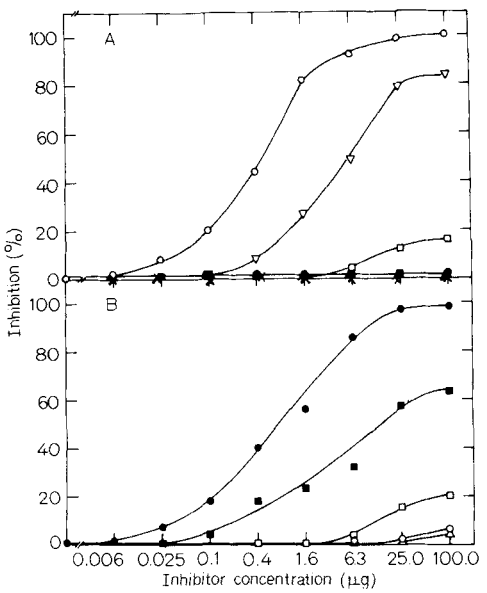

Fig. 2. *Radioimmunoassay of ferredoxin from different sources.* H-ferredoxin (○); *B. vulgaris* ferredoxin (●); lettuce ferredoxin (■); *Spirulina maxima* ferredoxin (□); adrenodoxin (×); *H. halobium* ferredoxin (▽). Each ferredoxin preparation was tested for inhibition in two systems: (A) ^{125}I-labelled H-ferredoxin and antiserum to H-ferredoxin (1:160); (B) ^{125}I-labelled *B. vulgaris* ferredoxin, and antiserum to *B. vulgaris* ferredoxin (1:100), percentage inhibition of each of the labelled ferredoxins to its antibodies was calculated from the binding value in the absence of inhibitor

ferredoxin. The same results were obtained with three different antisera to each immunogen.

The antigenic relationships between H-ferredoxin and ferredoxins from various sources including: *B. vulgaris*, lettuce, *Spirulina maxima*, bovine adrenal gland (adrenodoxin) and *H. halobium* were further investigated by inhibition studies. Each of the tested ferredoxins, in different amounts was mixed with either H-ferredoxin or *B. vulgaris* ferredoxin, labelled with ^{125}I. Subsequently, the respective homologous antiserum was added and the inhibition of binding of the radioactive ferredoxin to its antibodies was calculated. The results are shown in Fig. 2. Each of the ferredoxins used for immunization was effective in inhibiting the binding of the respective antiserum to the homologous ^{125}I-labelled ferredoxin. Inhibition was proportional to the amount of the ferredoxin in the range of 25 ng to 10 µg. The sensitivity could be further increased by preincubating the inhibiting ferredoxin, with the antiserum prior to the addition of the labelled antigen, but this sensitivity was not required in the present study. The antiserum against ferredoxin of *Halobacterium* from the Dead Sea did not react with the plant-type ferredoxins represented in this study by the proteins from lettuce and *B. vulgaris* and vice versa. This antigenic foreignness between these two types of ferredoxin is in agreement with the binding results shown in Fig. 1. The antigenic cross-reactivity between the ferredoxins of the *Halobacterium* of the Dead Sea and of *H. halobium* is considerable (Fig. 2A) and over 80% inhibition of antigen binding took place in the presence of excess of the latter. However, at least 20-fold higher concentrations of *H. halobium* ferredoxin were required for inhibition as compared to the amount of ferredoxin of *Halobacterium* of the Dead Sea. As for the plant-type ferredoxins, considerable cross-reactivity was noted between ferredoxins of lettuce and *B. vulgaris* (tested with ^{125}I-labelled *B. vulgaris* ferredoxin and its antiserum). As shown in Fig. 2B, over 60% inhibition could be obtained by high concentrations of the lettuce ferredoxin. Adrenodoxin, even at the highest concentrations tested, gave almost no inhibition in both the halophilic type (Fig. 2A) and the plant-type (Fig. 2B) systems. However, the ferredoxin from *Spirulina* maxima gave significant inhibition values (up to about 20%) in both systems, indicating that it bears antigenic determinants, part of which are present on the plant-type ferredoxin, and part on the halophilic ferredoxin.

Antigenic Relationships between Holo-H-ferredoxin and Apo-H-ferredoxin

Possible structural differences, manifested by alteration of antigenic properties, which may take place in the H-ferredoxin molecule upon removal of the iron-sulfur complex, were investigated.

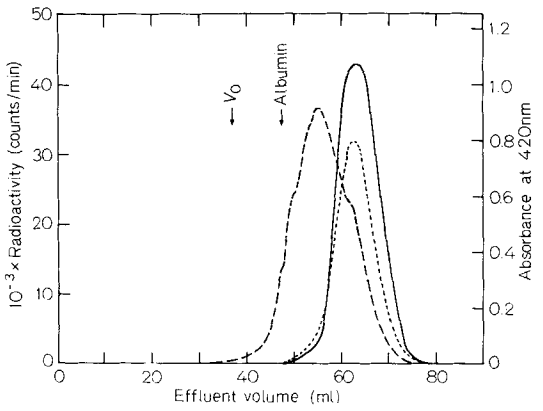

Fig. 3. *Gel filtration of holo-H-ferredoxin; and apo-H-ferredoxin and reduced and alkylated apo-H-ferredoxin on glass bead-filled column of Sephadex G-100.* The marker for the volume was dextran blue 2000. Radioactivity of (-----) apo-H-ferredoxin; (--------) reduced and alkylated apo-H-ferredoxin. (———) Absorbance at 420 nm, holo-H-ferredoxin

The apparent molecular size of the holo-H-ferredoxin as well as the two preparations of apo-H-ferredoxin mentioned above was determined by chromatography on a Sephadex G-100 column. Fig. 3 shows that upon removal of the iron-sulfur complex a change in the gel filtration pattern occurs, which indicates that the apo-protein has become aggregated into high molecular weight complexes. The reduced and alkylated H-ferredoxin, produced a protein peak which almost coincided with that of the holo-H-ferredoxin. The apo-H-ferredoxin as well as the reduced and alkylated apo-H-ferredoxin were iodinated and tested for binding by antisera towards either the apoprotein or the holoproteins. The results are shown in Fig. 4A and B respectively. Both antisera gave very similar binding patterns, although the antiserum to apo-ferredoxin had a considerably lower titer. The two antisera bound the holo-ferredoxin and the reduced and alkylated apo-protein to almost the same extent. The non-reduced apo-ferredoxin, however, was bound by the two antisera more strongly, almost by two orders of magnitude. It should be emphasized that the apo-ferredoxin, in the native, non-reduced state, has a higher apparent molecular weight, as shown in Fig. 3.

The inhibition studies depicted in Fig. 5, were performed with two systems: iodinated reduced and alkylated apo-H-ferredoxin was tested with antiserum to apo-ferredoxin and the iodinated holoferredoxin with its antiserum. As shown in the figure, the apoferredoxin (non-reduced) gives a considerably stronger inhibition pattern than that obtained with both the reduced and alkylated apoferredoxin and the holoferredoxin, which were practically indistinguishable. All antigens, when taken in excess yielded inhibition values of close to 100%.

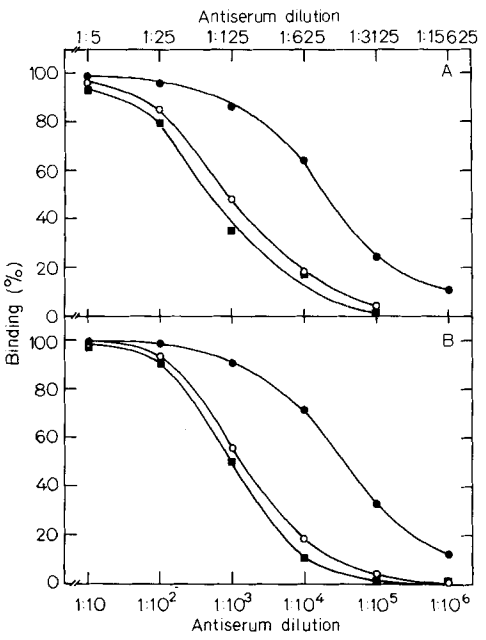

Fig. 4. *Antigen binding assay of H-ferredoxin (○); apo-H-ferredoxin (●); and reduced and alkylated H-ferredoxin (■) by antiserum to apo-H-ferredoxin, (A); or by antiserum to holo-H-ferredoxin (B)*

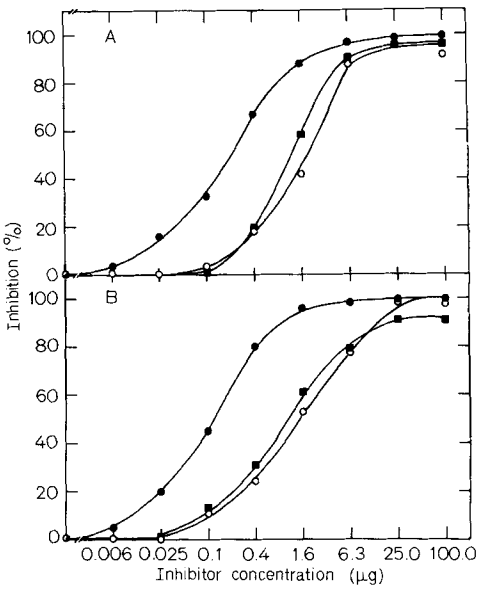

Fig. 5. *Radioimmunoassay of holo-H-ferredoxin (○); apo-H-ferredoxin (●); and reduced and alkylated-apo-H-ferredoxin (■).* Each preparation was tested for inhibition in two systems: ^{125}I-labelled reduced and alkylated apo-ferredoxin with antiserum to apo-ferredoxin (1:25), (A); ^{125}I-labelled holo-ferredoxin with antiserum to holo-ferredoxin (1:160) (B)

The Effect of Antiserum against H-Ferredoxin on Its Reducibility

The possibility that the interaction between H-ferredoxin and its antiserum may affect the redox properties of the protein was tested in two experimental systems: photoreduction of the ferredoxin (from *Halobacterium* of the Dead Sea or from *B.*

vulgaris) by lettuce chloroplasts, and chemical reduction with sodium dithionite (see Methods). In both cases the amount of antiserum used was sufficient to bind all the ferredoxin in the system, and control experiments with identical concentrations of non-immune serum (instead of anti-ferredoxin) were conducted in parallel. In both experimental systems, the antisera against H-ferredoxin did not have any apparent effect on the reducibility of the ferredoxin tested.

DISCUSSION

In the present study an immunochemical approach has been used for the comparison of ferredoxin isolated from *Halobacterium* of the Dead Sea, and ferredoxins of a variety of sources, as well as for the elucidation of the structural interrelationships between the holo-H-ferredoxin and its apo-derivative.

The methodology used by us throughout this study is based mainly on binding of the antigen (direct binding or competition studies) by the antibodies in contrast to the analysis by precipitin curves as performed by Tel-Or *et al.* [27]. We found this technique advantageous since it measures directly the interaction of antibodies with antigenic determinants of the ferredoxin molecule without requiring the formation of a precipitable complex. This is especially important in small protein antigens, which may express only very few antigenic determinants and thus will not form a very stable immune precipitate. Indeed, we have noticed that the precipitin reaction of H-ferredoxin with its antibodies either in solution or in agar gels was poor and the precipitin arcs, obtained after gel double immunodiffusion, dissociated after prolonged rinsing of the plates with $NaCl/P_i$. The antigen binding assay and the competition experiments also offer high sensitivity in the detection of antibody activity or antigen concentration, respectively.

Preliminary attempts to immunize rabbits with free H-ferredoxin resulted in the formation of only poor titers of anti-ferredoxin. Therefore all ferredoxin preparations which have been used for immunization were first coupled to the synthetic carrier multichain polyalanine, a procedure which resulted in an increase of the antibody titers in the immunized animals.

As stated above, the immunochemical data strongly support the existence of a class of 'halophilic-type ferredoxin', distinct from all other ferredoxins tested. The discrimination between the halophilic and the plant-type ferredoxin as evidenced by the immunochemical analysis, is especially meaningful, since several non-structural properties, including number of Fe-S groups in the molecule, optical properties, electron paramagnetic resonance, etc., seem to be similar in the two classes of ferredoxin. It should, however, be mentioned that the two halophilic ferredoxins tested were not antigenically identical and the ferredoxin from *H. halobium* (in excess) could inhibit no more than 80–85% of the interaction of H-ferredoxin with its specific antibodies. Although the halophilic and the plant-type ferredoxins exhibited no direct antigenic cross-reactivity with each other, some indirect evidence points to some structural relationships between the two classes. This was concluded from the finding that ferredoxin from *Spirulina maxima* cross-reacted significantly (up to about 20%) with both H-ferredoxin and with the plant-type (*B. vulgaris*) ferredoxin. The degree of antigenic cross-reactivity could not be related directly to defined structural differences between the tested ferredoxins, mainly since no detailed information on the number and nature of the antigenic determinants of H-ferredoxin is available at present. However, it has recently been shown [32] that a very high degree of similarity existed between the sequences of the *H. halobium* ferredoxin and that of the blue-green alga *Nostoc muscorum*. Thus, the antigenic cross-reactivity between the halophilic and algal ferredoxins was expected, since in general, the degree of antigenic cross-reactivity in a variety of protein antigens was found to be correlated to similarities in the sequences of the proteins under study [18,33]. The relations between primary sequence and the conformation of H-ferredoxin and its antigenic properties may become illuminated when the fine characterization of this molecule will be complete.

In the experiments with the apo-ferredoxins 3 proteins were tested: holo-ferredoxin, apo-ferredoxin, prepared under acidic conditions and the reduced and alkylated apo-ferredoxin. Both the antigen-binding assays and the competition experiments have indicated that the apo-ferredoxin and even its reduced and alkylated derivative contain all the antigenic determinants present in the holo-protein, as concluded from the fact that all the tested antigens could be either bound or alternatively could inhibit completely the binding of radioactive holo-H-ferredoxin by its antiserum.

In spite of the apparent similarity in the antigenic determinants of holo-ferredoxin versus apo-ferredoxins, clear differences were noted in the expression of antigenicity between the holo-protein and its reduced and alkylated apo-derivative on the one hand, and the apo-ferredoxin on the other hand. These differences were manifested by considerably higher binding values and competition capability as compared to those of the holo-ferredoxin. A similar phenomenon had been previously described for the ferredoxin from *Clostridium pasteurianum* by Nitz *et al.* [34]. They found that the performic-acid-oxidized ferredoxin reacts better than the holo-protein with antibodies raised against the latter, a phenomenon which was

attributed to differences in antigenic determinants on the two proteins. In the case of H-ferredoxin described above it seems that this explanation is unlikely and that the enhancement of antigenic activity of the apo-ferredoxin probably stems from the fact that upon removal of the prosthetic group, exposure of the free sulfhydryl groups of the active center takes place, which is followed by an aggregation of the apo-ferredoxin molecules. These aggregates may have a considerably higher antigenic valence than the monomeric protein and thus exhibit a much higher avidity towards the antibodies. The increase of either antibody or antigen valence has been shown (on both experimental and theoretical grounds to result in the elevation of the apparent binding constants by several orders of magnitude [35,36]. Thus, the aggregated protein may be bound more strongly than the monomer by the antibodies. This effect may be pronounced mainly in small protein antigens, such as ferredoxins, which contain only very few potent antigenic determinants. This explanation is also favored by the fact that upon reduction and alkylation both the molecular weight and the antigenic properties of the apo-ferredoxin were found to be essentially identical to those of the monomeric holo-ferredoxin. Our results with the holo-ferredoxin and the apo-ferredoxins have indicated that no antibodies specific to the prosthetic group could be found. This observation is corroborated by the fact that binding of ferredoxin by antibodies did not abolish its functional capacity to be reduced in two different experimental systems. The fact that prosthetic groups do not elicit the formation of specific antibodies had been reported for many other protein antigens [12].

The results described above point to the possibility of using immunochemical reagents for a detailed survey of the structural relationships between different 'halophilic' ferredoxins. The radioimmunoassay is being used at present for the quantitative determination of ferredoxin in the *Halobacterium* under a variety of growth conditions. It is anticipated that systematic analysis of the effect of antibodies to ferredoxin on different metabolic pathways in *Halobacterium* of the Dead Sea may shed light on the function of the ferredoxin in the metabolism of the bacteria.

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