BBA 45848

FERREDOXIN FROM THE PHOTOSYNTHETIC BACTERIUM, *CHLOROBIUM THIOSULFATOPHILUM*. A LINK TO FERREDOXINS FROM NONPHOTOSYNTHETIC BACTERIA

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(Received April 14th, 1969)

SUMMARY

1. Ferredoxin from the green photosynthetic bacterium, *Chlorobium thiosulfatophilum*, was purified and certain of its properties were determined. The purest preparations showed a ratio of $A_{385\ m\mu}/A_{280\ m\mu}$ of 0.71 and contained 4.5 iron and acid-labile sulfide groups per molecule.

2. Like ferredoxins from nonphotosynthetic anaerobic bacteria, Chlorobium ferredoxin showed a molecular weight of 6000 (based on amino acid composition and amino-terminal alanine) and lacked the full complement of amino acids. Chlorobium ferredoxin contained 13 different amino acids and was devoid of lysine, histidine, arginine, tryptophan and methionine. As for all ferredoxins examined, the amino-terminal end group of Chlorobium ferredoxin was alanine.

3. Chlorobium ferredoxin resembled *Chromatium* ferredoxin in its absorption spectrum (peaks at 385 and 280 mμ, shoulder at 300 mμ), in having glycine as C-terminal end group, and in its redox properties. Chlorobium ferredoxin was reduced photochemically with chloroplast suspensions but not with sodium dithionite.

4. The characteristics of Chlorobium ferredoxin are consistent with the view that it is a link between the ferredoxins of nonphotosynthetic anaerobic bacteria and those of photosynthetic bacteria of the *Chromatium*-type. The possible evolutionary significance of these findings is discussed.

INTRODUCTION

Ferredoxins are members of a family of iron–sulfur proteins which function as electron carriers in a variety of biological reactions[1–4]. Ferredoxins are widely distributed in nature and are characterized by an oxidation–reduction potential about equal to that of hydrogen gas (—420 mV at pH 7)[2,4]. Based on their absorption spectra, ARNON[1] has divided ferredoxins into two main types: a bacterial type, occurring in photosynthetic and nonphotosynthetic bacteria, and a plant type, occurring in algae and higher plants. The two types, as represented by ferredoxin

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from the nonphotosynthetic, anaerobic bacterium, *Clostridium pasteurianum*, and by ferredoxin from spinach chloroplasts, have a similar oxidation–reduction potential and are functionally interchangeable in certain biological reactions, such as the photoreduction of NADP by chloroplasts; but they differ in amino acid composition, in the number of iron–sulfur groups and, consequently, in molecular weight.

The occurrence of ferredoxin in nonphotosynthetic anaerobes[5], photosynthetic anaerobes[6,7], and green plants (including algae)[1] presents an opportunity to determine the probable evolutionary relationship of these groups of organisms, based on the characteristics of their respective ferredoxins. A comparison of the complete amino acid sequences of ferredoxins from nonphotosynthetic anaerobes of the Clostridium-type[8,9] and of ferredoxins from a green alga[10] and higher plants[11–14] suggests that, despite certain present differences in physical and chemical properties, clostridial and plant ferredoxins evolved from a common archetype[11,13].

The relationship of ferredoxins of photosynthetic anaerobes to the clostridial and plant ferredoxins is of particular interest since it may possibly give clues to the evolution of the photosynthetic apparatus of higher plants. The ferredoxin from photosynthetic bacteria most extensively investigated is that of *Chromatium*, which has an absorption spectrum[15] similar to the clostridial ferredoxins but a considerably different amino acid composition and molecular weight[16]. On the basis of its novel characteristics, SASAKI AND MATSUBARA[16] suggested that *Chromatium* ferredoxin may represent a third type, which is distinct from clostridial and plant-type ferredoxins.

We have now found that ferredoxin from the photosynthetic bacterium, *Chlorobium thiosulfatophilum*, is similar to clostridial ferredoxins in molecular weight, amino acid composition, and carboxy-terminal characteristics but resembles more closely *Chromatium* ferredoxin in absorption spectrum, redox properties and C-terminal end group. These findings are consistent with the view that Chlorobium ferredoxin is a link between the ferredoxins of nonphotosynthetic anaerobes and those of photosynthetic bacteria of the *Chromatium*-type.

METHODS

Chlorobium thiosulfatophilum cells (strain Tassajara) were grown and cell-free extracts were prepared as described previously[7]. Ferredoxin was isolated from cell-free extracts as described by BUCHANAN *et al.*[17], but as stated elsewhere[7], Chlorobium ferredoxin was not stable and lost sulfide on standing. The purification procedure was, therefore, carried out in a minimum time, usually within 2 days.

Determinations of iron, acid-labile sulfide and protein were carried out as described by LOVENBERG *et al.*[18]. The amino acid composition was determined as described previously[16] on 20- and 42-h hydrolysates. Half-cystine and methionine values were obtained from the 20-h hydrolysate of a performic acid-oxidized sample. Tryptophan was determined by the Ehrlich reaction[19]. The amino-terminal amino acid residue was determined by a modified Edman's phenylisothiocyanate method[20]. The phenylthiohydrantoin derivative of the amino acid was identified by paper chromatography[21]. Carboxypeptidase A was used to digest the carboxyl-terminal region of performic acid-oxidized ferredoxin according to FRAENKEL-CONRAT *et al.*[22]. Other sequence studies were carried out as described previously[16].

RESULTS

Spectral and redox properties of Chlorobium ferredoxin

Fig. 1 shows the absorption spectrum of Chlorobium ferredoxin. Like other bacterial ferredoxins, Chlorobium ferredoxin showed single absorption peaks in the visible (385 mμ) and ultraviolet (280 mμ) regions with a shoulder at 300 mμ. The absorption peak at 385 mμ is the same as for *Chromatium* ferredoxin and is displaced somewhat toward the blue end of the spectrum by comparison with the corresponding

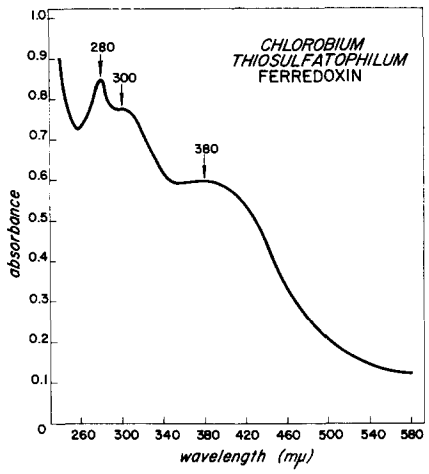

Fig. 1. Absorption spectrum of ferredoxin from *Chlorobium thiosulfatophilum*. The cuvette contained 0.25 mg of ferredoxin per ml in 0.30 M Tris buffer (pH 7.3), containing 0.54 M NaCl. The spectrum was measured in 1.0-cm cuvettes with a Cary 14 M recording spectrophotometer.

390 mμ peak in ferredoxins from nonphotosynthetic anaerobes. Our best preparations of Chlorobium ferredoxin showed an $A_{385\ m\mu}/A_{280\ m\mu}$ ratio of 0.71, which is similar to corresponding ratios of 0.74–0.80 observed for the clostridial[18] and *Chromatium*[15] ferredoxins.

Chlorobium ferredoxin was photochemically reduced with isolated spinach chloroplasts as described by BACHOFEN AND ARNON[15]. As with other ferredoxins, the absorption peak of Chlorobium ferredoxin in the visible region (385 mμ) disappeared upon reduction; the photochemically reduced ferredoxin could be reoxidized by oxygen. Chlorobium ferredoxin resembled the ferredoxins from other photosynthetic bacteria[15,23] in that it was not appreciably reduced by sodium dithionite. In this respect, the ferredoxins from all photosynthetic bacteria examined differ from those of *Clostridium pasteurianum* and chloroplasts[24].

Iron–sulfide content

As is true of other ferredoxins, Chlorobium ferredoxin contained equivalent amounts of iron and acid-labile sulfide. Based on protein determination by the phenol reagent assay, Chlorobium ferredoxin contained 0.75 μatom iron and 0.75 μmole sulfide per mg protein; these values correspond provisionally to 4.5 iron and sulfide groups per molecule, based on a molecular weight of 6000 (see below). The corresponding values are 6–7 iron and sulfide groups per molecule for ferredoxins isolated

from nonphotosynthetic bacteria[18], 5–6 for Chromatium ferredoxin[15,25], and 2 for algal[26] and plant ferredoxins[1,12]. However, the final determination of the number of iron–sulfide groups per mole of Chlorobium ferredoxin must await a determination of its extinction coefficient based on dry weight.

Amino-terminal end group and molecular weight

As is true of all ferredoxins examined, the amino-terminal end group of Chlorobium ferredoxin was alanine. As in *Chromatium* ferredoxin, the amino acid adjacent to alanine was leucine. Tyrosine occupied the third position from the amino terminus.

A calculation of the molecular weight, based on amino-terminal alanine, gave a value of 5800 for the protein in the oxidized state—in good agreement with the minimum molecular weight of 6000 obtained from amino acid composition (see below).

Carboxy-terminal characteristics

Carboxypeptidase A released equal amounts of isoleucine, valine, glutamine, and glycine after 2 h digestion, and a limited hydrolysis suggested that isoleucine was at the fourth position from the carboxyl terminus. In a separate experiment using performic acid-oxidized ferredoxin, a peptide was isolated from a thermolysin digest by two-dimensional paper chromatography and electrophoresis. The sequence of this peptide was Ile–Val–Gln–Gly·COOH, in agreement with the partial sequence de-

TABLE I

AMINO ACID COMPOSITION OF FERREDOXINS FROM *Chlorobium thiosulfatophilum, Clostridium tetanomorphum, Chromatium* AND SPINACH CHLOROPLASTS

Neg. stands for negligible. No distinction is made between aspartate and asparagine or glutamate and glutamine.

Amino acid	*Chlorobium thiosulfatophilum*			*Clostridium tetanomorphum*[18]	*Chromatium Strain* D[16,17]	*Spinach chloroplasts*[11]
	Hydrolysis time		*Nearest integer*			
	20 h	*42 h*				
Lys	neg.	neg.	0	0	2	4
His	neg.	neg.	0	0	2	1
Arg	neg.	neg.	0	0	2	1
Trp	0	0	0	0	0	1
Asp	3.09	3.26	3	7	8	13
Thr	3.60	3.71	4	2	6	8
Ser	2.01	2.25	2	4	4	7
Glu	7.27	7.88	7–8	6	16	13
Pro	3.73	3.77	4	3	5	4
Gly	4.63	5.14	4–5	4	5	6
Ala	10.98	11.43	11	7	3	9
Cys	6.80	—	7–8	8	9	5
Val	2.29	2.87	3	6	6	7
Met	0	0	0	0	1	0
Ile	3.08	3.72	4	5	6	4
Leu	0.91	0.93	1	1	3	8
Tyr	1.31	1.61	2	1	3	4
Phe	1.09	1.07	1	1	0	2
Total residues			53–56	57	81	97

duced from the carboxypeptidase experiment. Chlorobium ferredoxin is, thus, similar to *Chromatium* ferredoxin[27] in having glycine as the carboxy-terminal residue, but resembles *Clostridium acidi-urici* ferredoxin[37] in having glutamine and valine as the second and third residues from the carboxyl terminus.

Amino acid composition

Typical clostridial-type ferredoxins contain about 55 amino acid residues per mole and characteristically lack several different amino acids, in particular basic amino acids[18,28,8,9]. On the other hand, ferredoxins from *Chromatium*[16], a green alga[26,10] and higher plants[11–14] contain 81–97 amino acid residues per mole, comprising nearly the full complement of amino acids.

Table I shows the amino acid composition of Chlorobium ferredoxin in comparison with that of ferredoxins from *Chromatium*, *Clostridium tetanomorphum* (a nonphotosynthetic anaerobe similar to *Clostridium pasteurianum*) and spinach chloroplasts. Chlorobium ferredoxin, like clostridial-type ferredoxins, contained a minimum of 54–56 amino acid residues (corresponding to a minimum molecular weight of 6000) and showed a paucity of basic, and an abundance of acidic, amino acids. The amino acid content of Chlorobium ferredoxin is thus similar, in general, to all clostridial-type ferredoxins which have been examined, but Table I shows that Chlorobium ferredoxin in particular resembles *Clostridium tetanomorphum* ferredoxin (both ferredoxins contain thirteen different amino acids and are devoid of the 5 amino acids lysine, histidine, arginine, tryptophan and methionine). The amino acid composition of Chlorobium ferredoxin shows major differences from that of *Chromatium* and spinach, which respectively contain 16 and 17 different amino acids and are devoid of only 1 or 2 amino acids (*Chromatium* ferredoxin lacks tryptophan and phenylalanine; spinach ferredoxin lacks methionine).

DISCUSSION

The relationship of the photosynthetic bacteria to other photosynthetic cells has remained a matter of interest since MOLISCH[29] established that, unlike green plants, photosynthetic bacteria do not evolve oxygen. Photosynthetic bacteria require strictly anaerobic conditions and use hydrogen gas, reduced sulfur compounds or organic acids as physiological reductants for photosynthetic growth on carbon dioxide (see ref. 30).

GAFFRON[31,32] has proposed the hypothesis that photosynthetic (and chemoautotrophic) bacteria were relatively unimportant evolutionary sidelines that developed after the main line of autotrophic life (that is, photosynthesis of green plants) took over the earth. According to this view, bacterial photosynthesis would represent a degradation of plant photosynthesis and, on an evolutionary scale, photosynthetic bacteria would be remote from nonphotosynthetic anaerobes (such as the clostridia) which had probably evolved in a much earlier evolutionary period when the earth had a primitive atmosphere devoid of oxygen.

A different view, advanced by ARNON *et al.*[33,34] assigns to photosynthetic bacteria a key position in the evolution of photosynthesis. In this proposal, photosynthetic bacteria evolved from anaerobic bacteria devoid of chlorophyll by acquiring a capacity to form chlorophyll pigments while retaining their anaerobic mode of life.

Accordingly, the first photosynthetic organisms on earth would have been obligate chlorophyllous anaerobes that resembled photosynthetic bacteria such as Chlorobium and *Chromatium*. ARNON *et al.*[33,34] envisaged that bacterial photosynthesis, devoid of oxygen, was followed by algal and plant photosynthesis which added oxygen to the earth's atmosphere. This view was based mainly on the relative contribution to plant and bacterial photosynthesis of cyclic and noncylic photophosphorylation (the two processes of light-induced ATP production in photosynthetic organisms) and on certain similar metabolic features of the photosynthetic and nonphotosynthetic anaerobes.

The distribution of ferredoxins presents an opportunity, independent of other criteria, to assess the possible evolutionary relationships of nonphotosynthetic anaerobes, photosynthetic bacteria, algae and green plants. As in all proteins, the amino acid sequences of ferredoxins are a direct translation, according to the genetic code, of corresponding genes in cells. An analysis of the amino acid sequences of different ferredoxins, as in the case of cytochromes[35,36], may therefore reveal the extent to which their parent cells are related. As already mentioned, the amino acid composition and sequences of the clostridial and plant ferredoxins (including algae) indicate that ferredoxins from nonphotosynthetic anaerobes and green plants have arisen from a common archetype[11,13]. Ferredoxin from the photosynthetic bacterium *Chromatium* shows certain characteristics intermediate between clostridial and plant ferredoxins[16], but its amino acid sequence, although still incomplete, indicates that *Chromatium* ferredoxin is more like the clostridial type[27].

The findings reported in this communication provide additional evidence for linking photosynthetic and nonphotosynthetic anaerobes. Chlorobium ferredoxin had properties characteristic of both Clostridium and *Chromatium* ferredoxins and showed no features of the plant-type. A quantitative assessment (based on nucleotide base changes in specific codons) of the relation of Chlorobium ferredoxin to other bacterial ferredoxins (and to the plant-type) must await a determination of its complete amino acid sequence. However, the known similarities in Clostridium, Chlorobium and *Chromatium* ferredoxins (based on their amino acid composition and other chemical and physical properties which distinguish them from the plant-type), together with the evidence for homology among the bacterial and plant ferredoxins[11,13] (based on amino acid sequence studies), are in accord with the evolutionary sequence of ferredoxins proposed in Fig. 2. Fig. 2 suggests that the ferredoxins of photosynthetic anaerobes (derived from anaerobic ancestors devoid of chlorophyll) may, in turn,

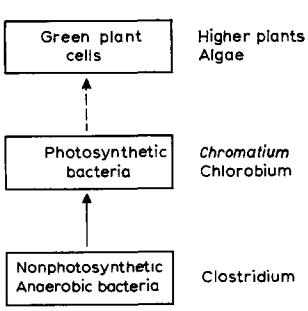

Fig. 2. Evolutionary development of ferredoxins.

have been the predecessors of ferredoxins of algae and higher plants, in general agreement with the view that ARNON *et al.*[33,34] proposed for the evolutionary development of photosynthesis. Fig. 2 represents the simplest possible scheme for the evolutionary relationships of ferredoxins. However, this interpretation is considered tentative and, pending further evidence, need not supersede the more detailed scheme previously proposed by MATSUBARA *et al.*[27].

The present finding that ferredoxin from the photosynthetic bacterium, *Chlorobium thiosulfatophilum*, is a link to clostridial ferredoxins raises the important question as to whether a corresponding link between the ferredoxins of photosynthetic bacteria and green plants has survived. No such ferredoxin has yet been described.

ACKNOWLEDGMENTS

This research was aided by grants from the National Institute of General Medical Sciences, the National Science Foundation and the Office of Naval Research to D. I. Arnon, and by grants from the National Aeronautics and Space Administration (NGR-05-003-020) and the National Institute of Health (HE-11553-02) to the Space Sciences Laboratory.

REFERENCES

1 D. I. ARNON, *Science*, 149 (1965) 1460.
2 A. SAN PIETRO AND C. C. BLACK, *Ann. Rev. Plant Physiol.*, 16 (1965) 155.
3 B. B. BUCHANAN, in C. K. JØRGENSEN, J. B. NEILANDS, R. S. NYHOLM, D. REINEN AND R. J. P. WILLIAMS, *Structure and Bonding*, Vol. 1, Springer, Berlin, 1966, p. 109.
4 R. MALKIN AND J. C. RABINOWITZ, *Ann. Rev. Biochem.*, 36 (1967) 113.
5 L. E. MORTENSON, R. C. VALENTINE AND J. E. CARNAHAN, *Biochem. Biophys. Res. Commun.*, 7 (1962) 448.
6 K. TAGAWA AND D. I. ARNON, *Nature*, 195 (1962) 537.
7 M. C. W. EVANS AND B. B. BUCHANAN, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 1420.
8 M. TANAKA, T. NAKASHIMA, A. BENSON, H. F. MOWER AND K. T. YASUNOBU, *Biochemistry*, 5 (1966) 1666.
9 J. N. TSUNODA, K. T. YASUNOBU AND H. R. WHITELEY, *J. Biol. Chem.*, 243 (1968) 6262.
10 K. SUGENO AND H. MATSUBARA, *Biochem. Biophys. Res. Commun.*, 32 (1968) 951.
11 H. MATSUBARA, R. M. SASAKI AND R. K. CHAIN, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 439.
12 S. KERESZTES-NAGY AND E. MARGOLIASH, *J. Biol. Chem.*, 241 (1966) 5955.
13 S. KERESZTES-NAGY, F. PERINI AND E. MARGOLIASH, *J. Biol. Chem.*, 244 (1969) 981.
14 A. M. BENSON AND K. T. YASUNOBU, *J. Biol. Chem.*, 244 (1969) 955.
15 R. BACHOFEN AND D. I. ARNON, *Biochim. Biophys. Acta*, 120 (1966) 259.
16 R. M. SASAKI AND H. MATSUBARA, *Biochem. Biophys. Res. Commun.*, 28 (1967) 467.
17 B. B. BUCHANAN, W. LOVENBERG AND J. C. RABINOWITZ, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 345.
18 W. LOVENBERG, B. B. BUCHANAN AND J. C. RABINOWITZ, *J. Biol. Chem.*, 238 (1963) 3899.
19 I. SMITH, *Nature*, 171 (1953) 43.
20 C. B. KASPER, H. MATSUBARA AND E. L. SMITH, *J. Biol. Chem.*, 240 (1965) 1131.
21 J. SJÖQUIST, *Acta Chem. Scand.*, 7 (1953) 447.
22 H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY, *Methods Biochem. Anal.*, 2 (1956) 397.
23 M. C. W. EVANS, *Biochem. Biophys. Res. Commun.*, 33 (1968) 146.
24 K. TAGAWA AND D. I. ARNON, *Biochim. Biophys. Acta*, 153 (1968) 602.
25 T. H. MOSS, A. J. BEARDEN, R. G. BARTSCH, M. A. CUSANOVICH AND A. SAN PIETRO, *Biochemistry*, 7 (1968) 1591.
26 H. MATSUBARA, *J. Biol. Chem.*, 243 (1968) 370.
27 H. MATSUBARA, T. H. JUKES AND C. R. CANTOR, *Brookhaven Symp. Biol.*, 21 (1968) 201.
28 B. B. BUCHANAN AND J. C. RABINOWITZ, *J. Bacteriol.*, 88 (1964) 806.
29 H. MOLISCH, *Die Purpurbakterien nach neuen Untersuchungen*, Fisher, Jena, 1907.
30 C. B. VAN NIEL, *Advan. Enzymol.*, 1 (1941) 263.

31. H. GAFFRON, *Proc. 5th Intern. Congr. Biochem., Moscow, 1961*, Vol. 3, MacMillan, New York, 1963, p. 114.
32 H. GAFFRON, in M. KASHA AND B. PULLMAN, *Horizons in Biochemistry*, Academic Press, New York, 1962, p. 59.
33 D. I. ARNON, F. R. WHATLEY AND M. B. ALLEN, *Science*, 127 (1958) 1026.
34 D. I. ARNON, M. LOSADA, M. NOZAKI AND K. TAGAWA, *Nature*, 190 (1961) 601.
35 E. L. SMITH, *Harvey Lectures*, 1967, p. 231.
36 C. NOLAN AND E. MARGOLIASH, *Ann. Rev. Biochem.*, 37 (1968) 727.
37 S. C. RALL, R. J. BOLINGER AND R. D. COLE, *Biochemistry*, 8 (1969) 2486.