Contrasting Evolutionary Histories of Chloroplast[1] Thioredoxins *f* and *m*

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Fourteen thioredoxin sequences were used to construct a minimal phylogenetic tree by using parsimony. The bacterial thioredoxins clustered into three groups: one containing the photosynthetic purple bacteria, *Escherichia* and *Corynebacterium;* a second containing the photosynthetic green bacterium, *Chlorobium;* and a third containing cyanobacteria. These groupings are similar to those generated from earlier 16s RNA analyses. Animal thioredoxins formed a fourth group. The two thioredoxins of chloroplasts (*f* and *m*) showed contrasting phylogenetic patterns. As predicted from prior studies, spinach chloroplast thioredoxin *m* grouped with its counterparts from cyanobacteria and eukaryotic algae, but, unexpectedly, thioredoxin *f* grouped with the animal thioredoxins. The results indicate that, during evolution, thioredoxin *m* of contemporary photosynthetic eukaryotic cells was derived from a prokaryotic symbiont, whereas thioredoxin *f* descended from an ancestral eukaryote common to plants and animals. The findings illustrate the potential of thioredoxin as a phylogenetic marker and suggest a relationship between the animal and *f*-type thioredoxins.

Introduction

Thioredoxins are a widely distributed group of small proteins, containing a highly conserved dithiol active site, that function in a number of cellular redox reactions (Holmgren 1985). Chloroplasts contain two types of thioredoxin (*f* and *m*) for which functional differences coincide with differences in structure (Buchanan 1980; Jacquot 1984; Cséke and Buchanan 1986; Maeda et al. 1986; Kamo et al. 1989). The thioredoxins, which are reduced photochemically by way of ferredoxin and ferredoxin-thioredoxin reductase, regulate biochemical activity in chloroplasts in response to light by chemically reducing specific target enzymes and thus changing their activities (Buchanan 1980; Jacquot 1984; Edwards et al. 1985; Leegood et al. 1985; Cséke and Buchanan 1986; Crawford et al. 1989). Thioredoxin *f* preferentially activates enzymes of the photosynthetic carbon cycle (fructose bisphosphatase, sedoheptulose bisphosphatase, phosphoribulokinase, NADP-glyceraldehyde 3-P dehydrogenase). Thioredoxin *m* preferentially activates an associated enzyme, NADP-malate dehydrogenase, and deactivates, also apparently by reduction, an enzyme of carbohydrate breakdown (glucose 6-P dehydrogenase). Both thioredoxins have the ability to interact with CF_1-ATPase, the enzyme forming ATP in chloroplasts. During the past year, the regulatory thiol site has been identified for the following thioredoxin-linked enzymes: fructose bisphosphatase (Marcus et al. 1988; Raines et al. 1988), NADP-malate dehydrogenase

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(Decottignies et al. 1988), phosphoribulokinase (Porter et al. 1988), and CF_1-ATPase (Miki et al. 1988). In each case, a specific disulfide group is reduced to the sulfhydryl level, leading to activation of the enzyme (Moroney et al. 1984; Crawford et al. 1989). This redox modulation enables chloroplasts to use light for the regulation of these enzymes, thereby achieving "biochemical order"—i.e., futile cycling is minimized so that starch can be built up in the light and broken down in the dark.

Comparisons of primary structure have revealed significant similarity between the *m*-type thioredoxins of chloroplasts and the thioredoxins from a variety of bacteria (e.g., see Maeda et al. 1986). Chloroplast thioredoxin *f*, by contrast, remains an enigma: nine residues are identical to those of other thioredoxins, but a phylogenetic relationship to bacterial or *m* thioredoxins seems quite distant (Kamo et al. 1989). Knowledge of the evolutionary history of thioredoxin *f* is, nevertheless, of interest because of the central role it plays in photosynthesis.

We have, therefore, attempted to gain information on the evolutionary history of chloroplast thioredoxin *f*. Our goal was first to establish the utility of thioredoxin as a phylogenetic marker, and, if it were found suitable, to deduce the evolutionary histories of the chloroplast thioredoxins. To this end, we have led an effort to determine the sequence of thioredoxins of several evolutionarily prominent bacteria (Johnson and Biemann 1987; Mathews et al. 1987; Johnson et al. 1988*b*), and we have now used this, as well as other, sequence information to construct phylogenetic (parsimonious) trees by computer analysis. A preliminary account of these findings has been published (Buchanan et al. 1989).

Material and Methods

Phylogenies were determined from the thioredoxin sequences by using the Macintosh 3.0 version of the PAUP ("phylogenetic analysis using parsimony") computer program (Swofford 1985). This program infers the relationships of protein sequences by using cladistic principles in which all of the extant sequences are assumed to be derived from a single hypothetical ancestral sequence (Felsenstein 1983, 1988). That tree considered most probable requires the smallest number of amino acid replacements needed to relate all of the extant sequences to the ancestral sequence. The 14 thioredoxin sequences were aligned manually. A few single internal amino acid gaps were introduced in the present analyses to increase the number of correct matches. An algorithm that performs pairwise alignments was used to guide the alignment of the entire set (Smith and Waterman 1981). Consistency indexes, which provide an indication of the amount of homoplasy or parallel evolution, were calculated using phylogenetically informative characters (Syvanen et al. 1989).

Results

Bacterial and m-Type Thioredoxins

The alignment of the 14 thioredoxin sequences analyzed in the present study is shown in figure 1. Visual inspection reveals that many positions are conserved among all of the thioredoxins, especially at their active site (W-C-G-P-C, positions 36–40). For the analyses described below, only the amino acids in positions 8–110 were utilized.

The phylogenetic pattern (minimal thioredoxin tree) deduced by analyzing the aligned bacterial and *m*-type thioredoxins from a variety of prokaryotes and photosynthetic eukaryotes is shown in figure 2. The bacteria analyzed range from photosynthetic bacteria (green sulfur *Chlorobium limicola* forma *thiosulfatophilum*, purple nonsulfur *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides*, and purple

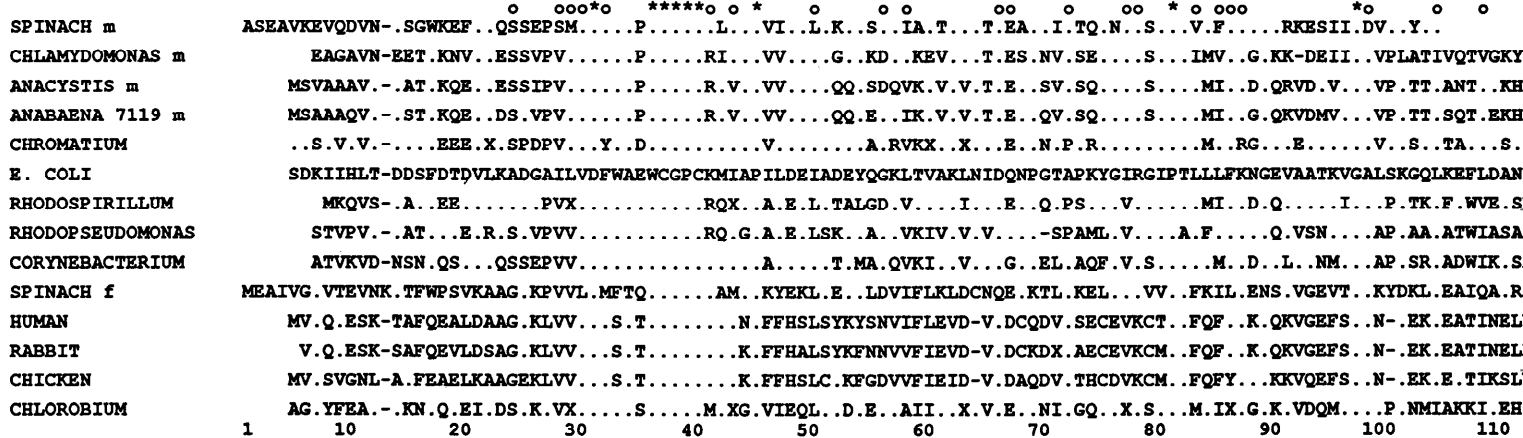

FIG. 1.—Aligned sequences of thioredoxins. The thioredoxin sequences have been published or are in press, with the exception of thioredoxin *m* from *Chlamydomonas reinhardtii* [Dr. P. Decottignies (Université de Paris-Sud), personal communication]. Sources for the sequences of the other thioredoxins are as follows: prokaryotes—*Anabaena* 7119 (Gleason et al. 1985), *Anacystis nidulans* R2 (Muller and Buchanan 1989), *Chlorobium limicola* forma *thiosulfatophilum* (Mathews et al. 1987), *Chromatium vinosum* (Johnson and Biemann 1987), *Rhodospirillum rubrum* (Johnson et al. 1988*b*), *Rhodopseudomonas sphaeroides* Y (Clement-Metral et al. 1988), *Corynebacterium nephridii* (Meng and Hogenkamp 1981), and *Escherichia coli* (Holmgren 1968, 1985; Mathews et al. 1987); photosynthetic eukaryote—spinach thioredoxin *m* (Maeda et al. 1986) and spinach thioredoxin *f* (Kamo et al. 1989); and animals—chicken (Jones and Luk 1988), rabbit bone marrow (Johnson et al. 1988*a*), and human lymphocyte (Wollmann et al. 1988). The indicated gaps were introduced to increase the number of matches. X = leucine or isoleucine in cases in which these amino acids were not distinguished. . = amino acid identical with that of *Escherchia coli* thioredoxin; * = amino acid identically conserved; ○ = amino acids functionally conserved with *E. coli* thioredoxin in all of the other thioredoxins examined.

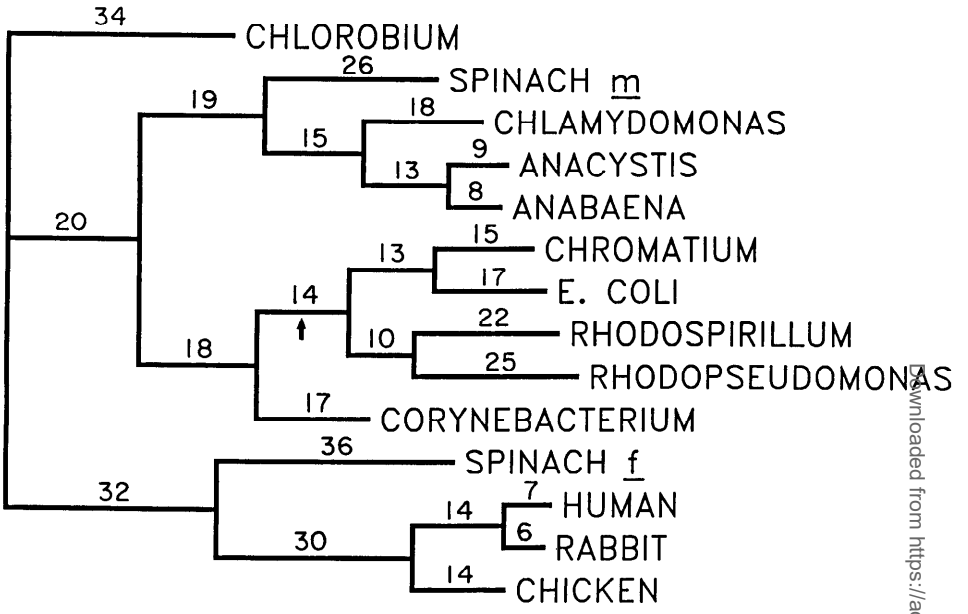

FIG. 2.—Parsimonious tree for prokaryotic and eukaryotic thioredoxins. Animal, higher-plant (*f* and *m*), algal (*m*), cyanobacterial (*m*), and bacterial thioredoxins are included. The numbers represent the number of amino acid replacements between the indicated thioredoxins. The vertical lines have no significance with regard to evolutionary distances. The arrow shows an alternative position for locating the animal and *f*-type thioredoxins that costs only one additional amino acid replacement. The tree shown was generated with a PAUP 3.0 computer program. The search was carried out using global branch swapping with MULPARS option in effect. The trees were unrooted. The total number of rearrangements was 960, and one parsimonious tree (shown above) of length 452 was found. The consensus tree generated by applying a bootstrap algorithm was the same as the parsimonious tree. The number of replicas was 200. The following parameters were used: starting seed was one, simple addition sequence, one tree held at each step, global branch swapping with MULPARS in effect, and trees were unrooted. In 98% of the replicas, spinach thioredoxin *f* was placed in the clade with the animal thioredoxins shown above.

sulfur *Chromatium vinosum*) to heterotrophic bacteria (the enteric *Escherichia coli* and filamentous *Corynebacterium nephridii*). The topology of the tree in figure 2 is exactly congruent with the topology of the tree based on 16s RNA that is given by Fox et al. (1980) and Woese (1987). That is, zero steps are required to convert one into the other by using the Waterman and Smith (1978) congruency test (for application of this test, also see Syvanen et al. 1989). The relationship of the oxygenic photosynthetic species to one another is also similar to that seen in the RNA studies—i.e., prokaryotic cyanobacteria (*Anabaena* 7119 and *Anacystis nidulans*) and the chloroplast of a eukaryotic alga (*Chlamydomonas reinardtii*) originate from a common ancestor. In the current case, thioredoxins from these photosynthetic species stem from a precursor that also gives rise to the chloroplast thioredoxin *m* of a higher plant (spinach). Recent evidence indicates that, in contrast to the situation in *E. coli* (Holmgren 1985), a functional thioredoxin *m* gene is required for growth of the cyanobacterium, *Anacystis nidulans,* an obligate phototroph (Muller and Buchanan 1989).

The thioredoxin *m* of oxygenic photosynthetic organisms resembles bacterial thioredoxin in activity, antigenicity, and primary structure (Schürmann et al. 1981; Gleason et al. 1985; Crawford et al. 1986; Cséke and Buchanan 1986; Muller and Buchanan 1989). Figure 2 shows that there is extensive phylogenetic information

stored in the sequences of these proteins. Whether this high content of phylogenetic information has been conserved in other major types of bacteria poses an interesting question, especially since *Methanobacterium thermoautotrophicum*—an archaebacterium—has been reported to contain a novel type of thioredoxin (Schlicht et al. 1985).

Thioredoxin *f*

What is the phylogenetic relationship between thioredoxin *f* and thioredoxin *m*? When we attempted to obtain a minimal tree by including spinach thioredoxin *f* with the bacterial and plant *m* thioredoxins, we found that thioredoxin *f* was highly diverged and placed in not one but in three equally possible, very different positions in the tree, which itself was unchanged (analysis not shown). Such behavior indicates that, despite invariant residues in the active site and other parts of the protein (Kamo et al. 1989), thioredoxin *f* is too different to group accurately with either bacterial or *m*-type thioredoxins—a finding in agreement with other data (Schürmann et al. 1981; Crawford et al. 1986). However, when we included animal thioredoxin in the analyses with thioredoxin *f*, a single cohesive picture emerged (fig. 2). Here, we found that chloroplast thioredoxin *f* (from spinach) grouped with animal rather than with bacterial or plant *m*-type thioredoxins. Furthermore, the pattern among the animal thioredoxins was also as expected—i.e., rabbit was close to human, and both were separated from chicken. Our phylogenetic analysis thus revealed a striking similarity between chloroplast thioredoxin *f* and the thioredoxin from animal cells. There has been little homoplasy or parallel evolution, as evidenced by the fact that the overall consistency index was 0.818 for the tree. We also explored alternative trees. The animal and chloroplast *f* thioredoxins could be placed on the branch with those of the purple bacteria, in a tree that is only one step longer than that of figure 2. This alternative topology is two nodes removed from that in figure 2, according to the Waterman and Smith congruency test. With this exception, the tree in figure 2 is robust.

Discussion

The finding that spinach thioredoxin *f*, a protein found in chloroplasts, is related to animal thioredoxins, while spinach thioredoxin *m*, also present in chloroplasts, is related to its counterpart in the cyanobacteria suggests the following chain of evolutionary events: Thioredoxin *f* is derived from a eukaryotic ancestor common to plants and animals, while thioredoxin *m* is descended from the photosynthetic symbiont that gave rise to the chloroplast. During the course of evolution, the thioredoxin *m* gene moved from the chloroplast to the nucleus and became adapted so that its product, a precursor form, could be transported into chloroplasts. The thioredoxin *f* gene of the nucleus became adapted to encode a form transportable into chloroplasts—a feature recently demonstrated experimentally by Kamo et al. (1989). The latter authors also noted a general similarity between the *f* and animal thioredoxins.

The current findings emphasize the need for new information in several areas. These include elucidation of the structure of thioredoxin *f* from additional organisms and clarification of the status of thioredoxin *f* in photosynthetic prokaryotes—i.e., cyanobacteria. The presence of *f*-like thioredoxin *activity* in the latter organisms (Yee et al. 1981) has been confirmed and extended, but the associated protein is atypically large (28 kDa), and its relation to thioredoxin *f* of higher plants is uncertain (Gleason 1984). The sequence of thioredoxin *h*, a component of the extrachloroplastic NADP/thioredoxin system of leaves (Florencio et al. 1988), may also give information on the

origin of thioredoxin *f*. The latter problem is timely because, if the present results linking chloroplast *f* and animal thioredoxins are confirmed and extended, it may be possible to gain new insight into the endosymbiotic history of the eukaryotic cells. Analysis of additional bacterial and chloroplast *m* thioredoxins would add to this history and possibly give an estimate of when endosymbiosis was established. Further characterization of the thioredoxin reported to occur in mitochondria is also warranted (Holmgren and Luthman 1978; Bodenstein-Lang et al. 1989).

The finding that thioredoxin *f* serves as a link between chloroplasts and animal cells raises another question: Does thioredoxin of animal cells play a primary regulatory role analogous to that of thioredoxin *f* in chloroplasts? The question is yet to be answered, but the recent demonstration that thioredoxin is induced in two systems—chicken fibroblasts following transformation by Rous sarcoma virus (Jones and Luk 1988) and human lymphocytes following activation of cell division (Wollmann et al. 1988)—suggests this to be a possibility.

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