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NADH:ubiquinone oxidoreductase from bovine heart mitochondria

A fourth nuclear encoded subunit with a homologue encoded in chloroplast genomes

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The amino acid sequence has been determined of the precursor of a nuclear encoded 20 kDa subunit of complex I from bovine heart mitochondria. The sequence of the mature protein is related to a protein of uncertain function, hitherto known as psbG, encoded in the chloroplast genomes of higher plants. Open reading frames encoding homologues of psbG have also been detected in bacteria and in the mitochondrial genome of *Paramecium tetraurelia*. The chloroplast *psbG* gene is found between *ndhC* and *ndhJ*, which encode homologues of ND3, a hydrophobic subunit of complex I encoded in the bovine mitochondrial genome, and of the nuclear encoded 30 kDa subunit of complex I. This 20 kDa protein is the eleventh out of the forty or more subunits of bovine complex I with a chloroplast encoded homologue, and its sequence provides further support for the presence in chloroplasts of a multisubunit enzyme related to complex I that could be involved in chlororespiration. The strict conservation of three cysteines suggests that the subunit might be an iron-sulphur protein.

Complex I (bovine heart mitochondria); Chloroplast genome homologue

1. INTRODUCTION

NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria is now known to be a multisubunit assembly of at least 41 different polypeptides [1,2]. Seven of them (known as ND1–ND6 and ND4L) are encoded in mitochondrial DNA [3–6], and the remainder are the products of nuclear genes that are imported into the organelle. Homologues of ND1–ND6 and of ND4L were found to be encoded in the chloroplast genomes of higher plants [7–9], and this observation led to the suggestion that chloroplasts contain an enzyme related to complex I, possibly an NAD(P)H:plastoquinone oxidoreductase involved in a chlororespiratory pathway, although such an enzyme has yet to be purified and characterized. The finding that three nuclear encoded components of mitochondrial complex I have chloroplast encoded homologues [10–12] provided additional support for the suggestion, and it is endorsed by the results described in the present paper. They show that a nuclear coded 20 kDa component of bovine complex I is related to another protein encoded in chloroplast genomes. This protein, known in the past as psbG, had been thought to be a component of photosystem II [13], but this was later shown to be incorrect [14]. It is now much more likely that psbG together with the other chloroplast encoded homologues of complex I is part of a chloroplast multisubunit complex related to complex I. The function of the mitochondrial subunit is not known. Its sequence contains three strictly conserved cysteines which might suggest that it is an iron-sulphur protein. This is consistent with the isolation of the protein from a preparation of a water-soluble fraction of bovine complex I, known as the iron-sulphur protein or IP fraction [15], and its presence in a sub-complex called Iα, which represents an extensive extramembrane domain containing the NADH binding site, FMN and all of the Fe-S clusters that have been detected by EPR (M. Finel, J.M. Skehel, S.J. Albracht and J.E. Walker, unpublished work).

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no. X65020.

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2. MATERIALS AND METHODS

2.1. *Isolation and protein sequencing of the bovine PSST protein*

Complex I was purified from bovine heart mitochondria [16]. Its subunits were separated on 2-dimensional gels by isoelectric focussing and gel electrophoresis in the presence of sodium dodecyl sulphate. The isoelectric focussing was conducted as described before [12], and then the cylindrical gel was placed on a 16.5% polyacrylamide gel prepared in urea according to Schägger and von Jagow [17], and embedded in a 4% stacking gel. The separated subunits were transferred to a poly(vinylidene difluoride) membrane and sequences were determined at their N-terminals. Sub-complex Iα was prepared by ion exchange chromatography on a Mono Q column (10 cm × 1 cm i.d.) from complex I that had been partly dissociated with the detergent LDAO (lauryldimethylamine oxide). This procedure will be described in detail by M. Finel, J.M. Skehel, S.J. Albracht and J.E. Walker (in

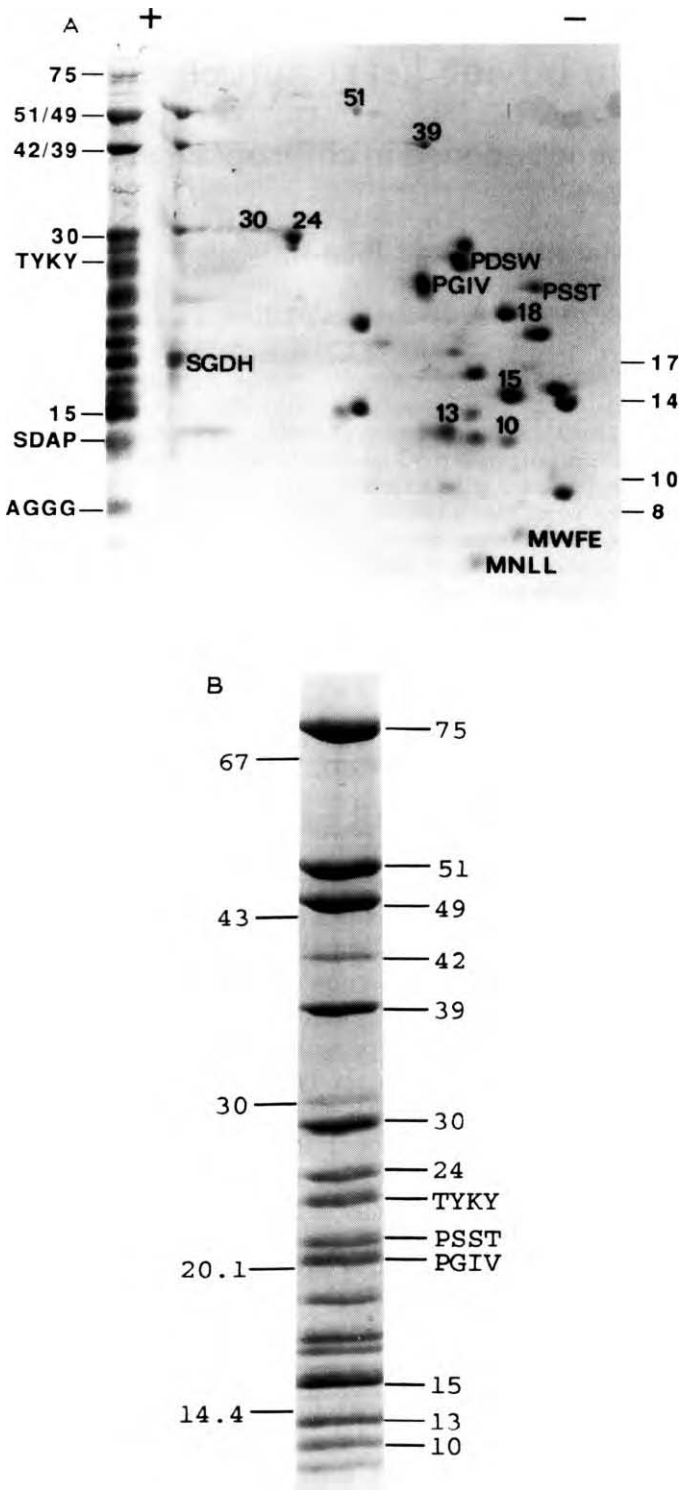

Fig. 1. Separation of the subunits of complex I from bovine heart mitochondria. (A) 2-Dimensional isolectric focussing (horizontal direction) and polyacrylamide gel electrophoresis in the presence of SDS of complex I. The positions of the anode and cathode in the isoelectric focussing are denoted by + and −, respectively. (B) Polyacrylamide gel electrophoresis in the presence of SDS of bovine sub-complex Iα. The positions in both gels of various subunits detected by N-terminal sequencing are indicated. The positions of various molecular weight markers are shown at the side of (A) and (B).

preparation). Its subunits were separated under denaturing conditions in a 10–25% polyacrylamide gradient gel [18], and N-terminal sequences were determined as described above.

2.2. *Amplification and sequencing of cDNA clones for the PSST subunit*

cDNAs for the PSST subunit were amplified from total bovine heart cDNA by the polymerase chain reaction (PCR) using, in the first instance, mixtures of synthetic oligonucleotides as primers and hybridization probes. These mixtures were based upon the N-terminal sequence of the protein (see above), and on published sequences of tryptic peptides [15]. Further details are given in the legend to Fig. 2. Similar experiments to amplify cDNAs for other subunits of complex I and their DNA sequence analysis are described elsewhere [2]. The sequence was determined in both senses of the DNA.

3. RESULTS AND DISCUSSION

3.1. *Sequence of the bovine PSST subunit*

Subunit PSST was detected in complex I by N-terminal sequence analysis from residues 1–15, and more recently it was also found in preparations of sub-complex Iα (see Fig. 1). On denaturing gels it migrates to a position between subunits TYKY and PGIV [12,19], and its apparent molecular weight is about 20 kDa. Subunit PSST has also been isolated by chromatography from a preparation of the IP fraction of bovine complex I [15], although it has not been detected in

Table I

Amino acid composition and molecular mass of the PSST subunit of bovine complex I

Amino acid	Number of residues	
	By analysis[1]	From sequence
Aspartic acid	11.3[2]	8
Asparagine	–	3
Threonine	5.0	5
Serine	12.1	13
Glutamic acid	11.7[3]	5
Glutamine	–	6
Proline	14.3	15
Glycine	10.5	11
Alanine	20.8	18
Valine	18.6	18
Methionine	7.8	9
Isoleucine	7.0	7
Leucine	12.4	12
Tyrosine	10.4	11
Phenylalanine	3.4	3
Histidine	2.0	2
Lysine	8.0	8
Arginine	15.5	17
Cysteine	5.2	5
Tryptophan	n.d.	3
total	–	179
molecular mass		20,077.5[4]

[1]quoted from [15]; [2]sum of aspartic acid and asparagine residues; [3]sum of glutamic acid and glutamine residues; [4]calculated from the sequence.

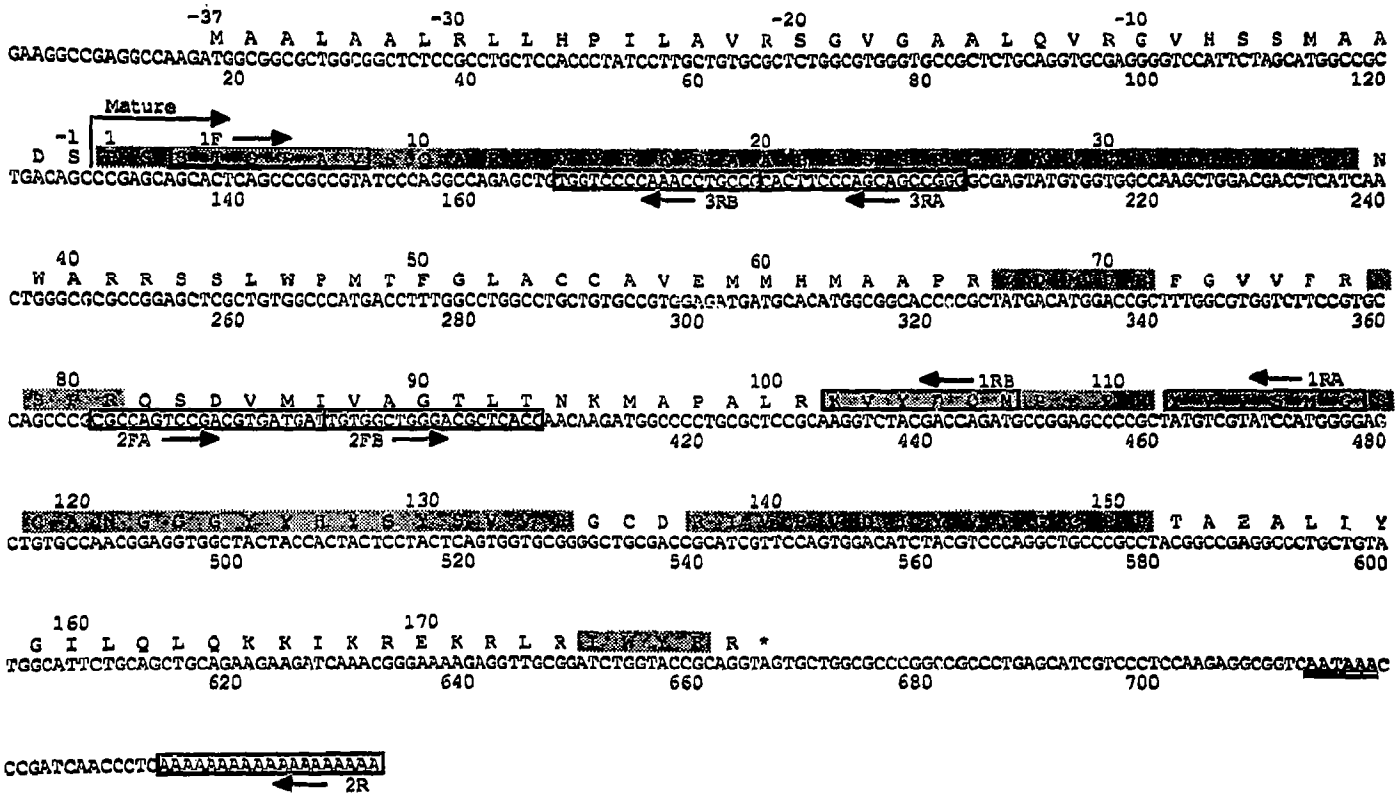

Fig. 2. Analysis by protein and DNA sequencing of the PSST subunit of complex I from bovine heart mitochondria. The shaded regions were determined by direct protein sequencing of the intact protein (residues 1–28) and tryptic peptides [15]. Overlapping cDNAs were isolated from total bovine cDNA by a strategy based on the polymerase chain reaction. The boxed protein sequences 1F, 1RA and 1RB were used to design synthetic oligonucleotide mixtures which were employed as forward and reverse primers, respectively, in the initial polymerase chain reaction. That based on 1F was the forward primer and those based on 1RA and 1RB were used as nested reverse primers. The boxed nucleotide sequence served as unique primers in two further reactions in which the sequence was extended to the 3′ and 5′ extremities. Nested forward primers 2FA, 2FB were used with reverse primer 2R in the former reaction, and an oligonucleotide complementary to a synthetic homopolymer 5′ G tail, which had been added to the cDNA [12], was used with nested primers 3RA and 3RB in the latter reaction. The senses of the various primers are indicated by arrows. A potential polyadenylation signal is underlined twice.

other independently prepared samples of the same fraction (J.E. Walker et al., unpublished observations).

Sequences of tryptic peptides (see legend to Fig. 2) isolated from the chromatographically purified subunit and the N-terminal sequence from residues 1–28 have been published [15]. Synthetic mixtures of oligonucleotides based on amino acids 3–8 and on the sequences KVYDQM and YVVSMG, present in tryptic peptides and now known to be amino acids 102–107 and 112–117, respectively, were used as primers in a polymerase chain reaction with total bovine cDNA as template. In the products of the reaction, a partial cDNA was detected by its hybridisation with a third mixture of oligonucleotides based on amino acids 10–15. This cDNA was found to encode amino acids 3–107 of the complete protein. The partial sequence was extended to the 5′ and 3′ extremities of the intact cDNA in two further polymerase chain reactions (see Fig. 2). The complete cDNA, compiled from the partial cDNA sequences, encoded a precursor of the PSST subunit, the mature protein sequence being preceded by a sequence 37 amino acids in length. Such presequences are common to many, but by no means all, nuclear encoded mitochondrial proteins, and direct them into the organelle. The presequence is removed during the import process. As in the case of the PSST protein, a net positive charge is a characteristic feature of such presequences [20], but the presence of an aspartic acid residue at position −2 is very unusual. More commonly, an arginine residue is found in the vicinity of the processing site, but is absent from this region of the precursor of the PSST subunit. The mature protein is 179 amino acids long, and all of the sequences determined by direct protein sequencing are present (Fig. 2). The molecular weight (20,077.5) and amino acid composition calculated from the sequence (see Table I) are both in excellent agreement with the experimentally determined values.

3.2. *Relationship of subunit PSST to protein sequences encoded in chloroplast genomes*

The sequence of the PSST subunit of bovine complex

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                                    20                  40                  60
B. taurus                    PSSTQPAVSQARAVVPKPAALPSSRGEYVVAKLDDLINWARRSSLWPMTFGLACCAVEM
P. tetraurelia                                      MILKADFLKLSANNLISWARQGSFWPLTFGLACCALEM
M. polymorpha          MVLNFKFFTCENSLEDNSTTMLKNSIESSFINKTLTNSIILTTFNDFSNWARLSSLWPLLYGTSCCFIEF
N. tabacum             MVLAPEYSDNKKKNGKNKIETVMNSIQFPLLDRTTQNSVISTTLNDLSNWSRLSSLWPLLYGTSCCFIEF
Z. mais                 MVLTEYSEKKKKEGKDSIETIMSLIEFPLLDQTSSNSVISTTPNDLSNWSRLSSLWPLLYGTSCCFIEF
O. sativa               MVLTEYSDKKKKEGKDSIKTVMSLIEFPLLDQRSSNSVISTTLKDLSNWSRLSSLWPLLYGTSCCFIEF
T. aestivum             MVLTEYLDKKK-EGKDSIETVMNLIEFPLLDQTSSNSVISTTPNDLSNWSRLSSLWPLLYGTSCCFIEF
Synechocystis   sp.       MSPNPANPTDLERVATAKILNPASRSQVTQDLSENVILTTVDDLYNWAKLSSLWPLLYGTACCFIEF
E. coli  fhl                        MSNLLGPRDANGIPVPMTVDESIASMKASLLKKAKRSAYVYRVDCGGCNGCEIEI
                                                                              *   *   *

                                80                  100                 120                 140
B. taurus              MHMAAPRYDMDRFGVVFRASPRQSDVMIVAGTLTNKMAPALRKVYDQMPEPRYVVSMGSCANGGGYYH-Y
P. tetraurelia         MHATVSRYDFDRFGVIFRATPRQADLIIVAGTVTNKMAPALRRLYDQTADPKWVLSMGSCANGGGYYH-Y
M. polymorpha          ASLIGSRFDFDRYGLVPRSSPRQADLIITAGTVTMKMAPSLVRLYEQMPEPKYVIAMGACTITGGMFSTD
N. tabacum             ASLIGSRFDFDRYGLVPRSSPRQADLILTAGTVTMKMAPSLVRLYEQMPEPKYVIAMGACTITGGMFSTD
Z. mais                ASLIGSRFDFDRYGLVPRSSPRQADLILTAGTVTMKMAPSLVRLYEQMPEPKYVIAMGACTITGGMFSTD
O. sativa              ASLIGSRFDFDRYGLVPRSSPRQADLILTAGTVTMKMAPSLVRLYEQMPEPKYVIAMGACTITGGMFSTD
T. aestivum            ASLIGSRFDFDRYGLVPRSSPRQADLILTAGTVTMKMAPSLVRLYEQMPEPKYVIAMGACTITGGMFSTD
Synechocystis   sp.    AALIGSRFDFDRFGLVPRSSPRQADLIITAGTITMKMAPALVRLYEEMPEPKYVIAMGACTITGGMFSSD
E. coli  fhl           FATLSPLFDAERFGIKVVPSPRHADILLFTGAVTRAMRSPALRAWQSAPDPKICISYGACGNSGGIFH-D
                              *  * *      **  *     *  *                 *      * *    *

                                        160                 180                 200
B. taurus              SYSVVRGCDRIVPVDIYVPGCPPTAEALLYGILQLQKKIKREKRLRIWYRR
P. tetraurelia         SYAVVKGCDKIIPVDMLCPRCPPTAEALFFGVLQLQKTLMKTINEKKVF
M. polymorpha          SYTTVRGVDKLIPVDIYLPGCPPKPEAIIDAIIKLRKKIAQEIYEEKKI--LKKGTRFFTLNHQFNFFSN
N. tabacum             SYSTVRGVDKLIPVDVYLPGCPPKPEAVIDAITKLRKKISRELYEDRIR--SQRANRCFTTNHKFHVQHS
Z. mais                SYSTVRGVDKLIPVDVYLPGCPPKPEAVIDALTKLRKKIAREIIEDRTLCQSQKKNRSFTTRHKLYVRRS
O. sativa              SYSTVRGVDKLIPVDVYLPGCPPKPEAVIDALTKLRKKISREIVEDRTL--SQKKNRCFTTSHKLYVRRS
T. aestivum            SYSTVRGVDKLIPVDVYLPGCPPKPEAVIDALTKLRKKISREIVEDRTL--SQNKNRCFTTSHKLYVRRS
Synechocystis   sp.    STTAVRGVDKLIPVDVYIPGCPPRPEAIFDAIIKLRKKVANESIQERAI--TQQTHRYYSTSHQMKVVAP
E. coli  fhl           LYCVWGGTDKIVPVDVYIPGCPPTPAATLYGFAMALGLLEQKIHARGPGELDEQPAEILHGDMVQPLRVK
                             * *   *** * * ***   *

                                  220                 240
M. polymorpha          LDNPKLTSSNQFFQSKKTSKVLLETSLTFKEKENL
N. tabacum             IHTGNYDQRVLYQPPSTSEIPTEIFFKYKNSVSSPELVN
Z. mais                THTGTYEQELLYQSPSTLDISSETFFKSKSSVSSYKLVN
O. sativa              TNTGTYEQELLYQSPSTLDISSETFFKSKSPVSSYKLVN
T. aestivum            THTGTYEQELLYQSPSTLDISSETFFKSKSSVPSYKLVN
Synechocystis   sp.    ILDGKYLQQGTRSAPPRELQEAMGMPVPPALTTSQQKEQLNRG
E. coli  fhl           VDREARRLAGYRYGRQIADDYLTQLGQGEEQVARWLEAENDPRLNEIVSHLNHVVEEARIR
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Fig. 3. Comparison of the sequence of the PSST subunit of bovine complex I with related protein sequences. The sequences shown are encoded in mitochondrial DNA of *Paramecium tetraurelia* [31,32], the chloroplast genomes of liverwort (*Marchantia polymorpha*), tobacco (*Nicotiana tabacum*), maize (*Zea mais*), rice (*Oryza savita*) and wheat (*Triticum aestivum*) [7–9,14] and the genome of the cyanobacterium, *Synechocystis* sp. PCC 6803 [26]. *E. coli* fhl denotes a protein encoded in an open reading frame in a locus associated with formate hydrogen lyase [29]. Conserved amino acids are marked with an asterisk.

I is related to a protein of unknown function found in the chloroplast genomes of higher plants, and homologues have also been found in bacteria and in the mitochondrial genomes of *Paramecium tetraurelia* (see Fig. 3). The chloroplast protein was proposed to be a component of photosystem II [13], and in consequence it has been known as psbG. Subsequently, antibodies were prepared against the wheat chloroplast protein expressed in *E. coli*, but the distribution of the protein detected by the reagent did not correlate with that of photosystem II in various fractions of thylakoid membranes from pea chloroplasts. Therefore, it appeared that the original assignment was not correct [14]. The homology with subunit PSST of bovine complex I supports this view. It is proposed that *psbG* should be known instead as *ndhK*.

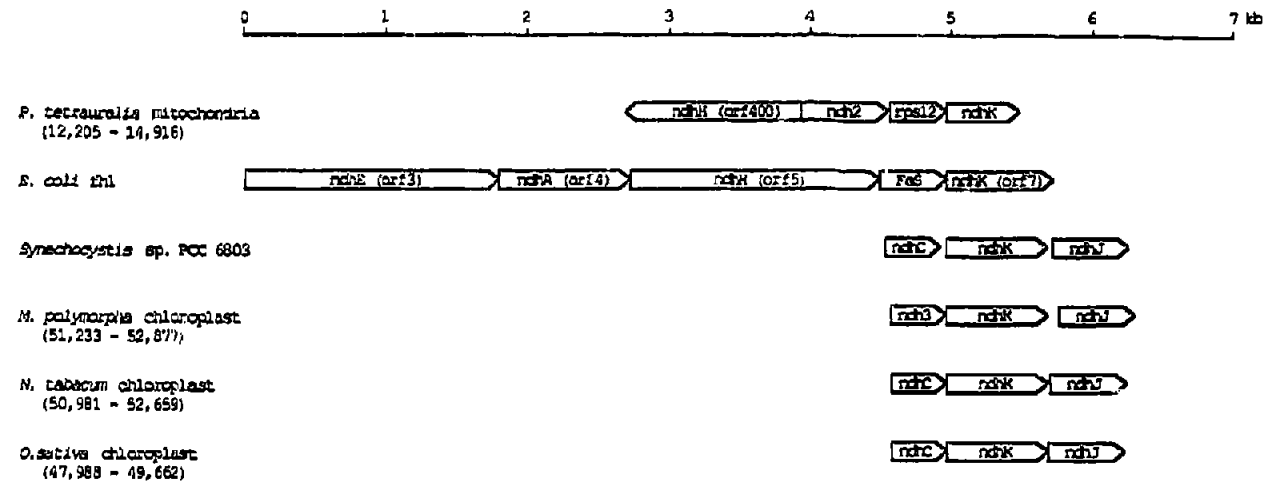

Fig. 4. Clusters of genes encoding homologues of complex I subunits in various species. *ndhA*, *ndhB*, *ndhC* and *ndhD* are the homologues of the genes for ND1, ND2, ND3 and ND4, respectively, four hydrophobic membrane subunits of complex I first found in the mitochondrial genomes of mammals [3–6]. *ndhH*, *ndhJ* and *ndhK* encode homologues of three nuclear encoded subunits of complex I, namely the 49 kDa subunit [10], the 30 kDa subunit [11] and subunit PSST (described in this paper). *ndhK* was formerly known as *psbG*. In *M. polymorpha ndh3* is the equivalent of *ndhC* in other species. rps 12 is a mitochondrial ribosomal component in *P. tetraurelia* [31,32], and FeS denotes a potential iron-sulphur protein of unknown function in *E. coli* [29]. The figure is redrawn from sequence data published in references [7–9,29,31,32]. The arrows at the end of each gene indicate the direction of transcription. The scale is in kilobases.

Eleven subunits of bovine complex I have now been found to have homologues encoded by chloroplast DNA [3–12], and the relationship between bovine PSST and ndhK provides further support for the view that chloroplasts contain an enzyme complex similar to mitochondrial complex I, possibly an NAD(P)H:plastoquinone reductase involved in chlororespiration. This term refers to an electron transport chain operating in the dark and under aerobic conditions at about 10% of the rate of respiration in mitochondria [21], in which electrons from NADH, NADPH, and also succinate [22], flow into the plastoquinone pool, and thence possibly to an unidentified terminal oxidase. Its physiological function in thylakoid membranes is unclear, but a number of suggestions have been advanced [23]. They include the maintenance of a pH gradient to keep ATPase in a functionally ready state in the event of a dark–light transition [21], more efficient carbon dioxide fixation on re-illumination by poising the reduction level of the photosynthetic apparatus under anaerobic or microanaerobic conditions, resulting in improved growth or survival in extreme conditions [24], the re-use of reducing equivalents produced by starch degradation to regenerate electron acceptors for triose phosphate formation [25], and contribution to acetate metabolism in the dark [21].

In chloroplast genomes, and also in the cyanobacterium *Synechocystis* sp. PCC 6803, *psbG* is found in association with genes encoding homologues of two other subunits of complex I [26] (see Fig. 4), and this led to earlier suggestions that psbG was related to an unidentified component of complex I [27,28]. In addition, psbG and ndhJ (the homologue of the 30 kDa subunit of bovine complex I) were detected by immunological means in the same crude enzyme fraction [28]. Although the suggestion that psbG is a homologue of a complex I subunit has now been proven to be correct, the association of *psbG* with other genes related to complex I components is rather weak evidence in favour of the proposal. After all, there are many other such associations of genes for proteins that do not co-assemble and have no obvious common biochemical features; the association of the gene for the ribosomal protein rps12 with *ndhH*, *ndh2* and *psbG/ndhK* in the mitochondrial DNA of *P. tetraurelia* (see Fig. 4) is one of numerous examples that could be cited.

An *ndhK* homologue and genes for homologues of other components of complex I have also been found clustered at a locus coding for components of *E. coli* formate hydrogen lyase [29]. This suggests that parts of this enzyme and of complex I have a common evolutionary origin, and that their present day structures and mechanisms retain common features. Three other subunits of complex I have been shown to be closely related to part of an NAD^+ reducing hydrogenase [30].

A final feature deserving comment is that three cysteine residues in the sequences of the ndhK proteins are conserved throughout the range of species that have been investigated, and in addition, a fourth cysteine is present in all but the *E. coli* formate hydrogen lyase ndhK protein (see Fig. 3). The roles of these conserved residues are not known, but one possibility is that they are involved in liganding one of the several Fe-S clusters that are known to be present in the enzyme (see [1] for a summary). Both binuclear and tetranuclear cluster types are usually bound by four cysteine ligands, but

other amino acids can also perform this function. Three of the cysteine ligands of tetranuclear clusters are often found in the sequence motif Cys*XX*Cys*XX*Cys, and the fourth cysteine is followed by proline. The sequence CysPro is conserved in PSST, but its conserved cysteines are well separated from each other. So even if PSST proves to be an Fe-S protein, the cluster type is not predicted by the sequence.

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