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IDENTIFICATION AND CHARACTERIZATION OF THE ctaC (coxB) GENE AS PART OF AN OPERON ENCODING SUBUNITS I, II, and III OF THE CYTOCHROME *c* OXIDASE (CYTOCHROME aa_3) IN THE CYANOBACTERIUM *SYNECHOCYSTIS* PCC 6803

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SUMMARY: The gene (coxII = coxB = ctaC) encoding subunit II of *Synechocystis* PCC 6803 cytochrome c oxidase has been isolated by screening a genomic DNA library in pUC18 with a 17-bp oligonucleotide probe (probe C) derived from coxI of *Paracoccus denitrificans* after Southern blots with a 19-kb oligonucleotide (probe A) derived from coxII of *P. denitrificans* had given equivocal results. A 2.2 kb PstI-KpnI restriction fragment was subcloned into pUC 18 and the resulting plasmid pDAUV26, which contained the probe C-binding site near the downstream end was found also to contain the whole coxII gene upstream of this site. The novel plasmid pDAUV 26 was used to transform competent *E. coli* cells, propagated therein, and the sequence determined. The 2.2 kb insert contained the entire coding region for the coxII gene together with a GAG start codon, a TAA stop codon, and a putative Shine-Dalgarno sequence. The deduced COII polypeptide is composed of 319 aa (calculated molecular mass of 32,800) plus a N-terminal leader sequence of 20 aa. The hydropathy plot suggests two lipophilic transmembrane domains near the N-terminus connected with an extremely hydrophilic aa stretch on the cytosolic side, while an unusually long (>50 aa) aa stretch on the periplasmic (= intrathylakoidal) side leads to a typical cyanobacterial threonine in place of the first conserved glutamate of the cytochrome c-binding region in all other COII proteins. Together with a considerably shortened and interrupted aromatic aa stretch in this region, these differences are discussed in terms of the peculiar affinity of cyanobacterial cytochrome oxidases for acidic *c*-type cytochromes. Other invariant features such as the strictly conserved Cu_A-binding aa, however, are found in correct positions.

It has been reported before that both plasma and thylakoid membranes isolated and purified from the closely related cyanobacterial strains *Synechocystis* PCC 6714 [1, 2] and PCC 6803 [2, 3] contain immunologically cross-

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Abbreviations: aa, amino acid(s); ctaC = coxII = coxB, gene encoding the cytochrome *c* oxidase subunit II polypeptide (COII protein); cyt, cytochrome; PCC, Pasteur Culture Collection, Paris (France); nt, nucleotide(s); bp, base pair(s); kb, kilo-base pair (thousand base pairs).

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reactive aa_3-type cytochrome *c* oxidase. Alike to many other cyanobacteria investigated so far [2, 4-8] spectral and kinetic properties, and inhibition profiles, of the membrane-bound enzyme were strikingly similar to those of the well-known enzymes from *Paracoccus denitrificans* or mammalian mitochondria (EC 1.9.3.1). Recently we were able to isolate and purify a four-subunit cytochrome *c* oxidase from plasma membrane preparations of *Anacystis nidulans* showing immunological, spectroscopic and kinetic properties in clear conformity with an aa_3-type cytochrome *c* oxidase [9, 10]. This prompted us to identify and characterize the genes encoding the enzyme in the well-known transformable cyanobacterium *Synechocystis* PCC6803 [11] starting with the ctaC or coxII or coxB gene coding for the subunit II (COII) polypeptide as to be detailed in this communication.

MATERIALS AND METHODS

Synechocystis PCC 6803 cells were picked as single colonies from BG-11 agar plates (medium BG-11, Ref. 12 plus 1,5 % Bacto agar) and grown in 100 ml Erlenmayer flasks containing 50 ml medium BG-11 in an illuminated New Brunswick shaker at 30-32°C (2 x 15 W fluorescent lamps). Stock cultures on BG-11 agar plates were grown in a Heraeus HPS 500 chamber at 32°C and 80 % humidity. *E. coli* HB 101 was used for large-scale production of plasmids [13]. *E. coli* JM 109 was used for the preparation of single stranded DNA [14]. Restriction endonucleases were obtained from Boehringer, Mannheim (FRG). Chromosomal DNA from cyanobacteria was prepared according to standard techniques [15]. The oligonucleotide probes were kindly donated to us by Dr. M. Saraste, Helsinki (Finland) and radioactively labeled as described in [16]. Southern blotting was performed according to the Amersham booklet "Membrane transfer and detection methods", Amersham, 1985. Rapid plasmid isolation from *E. coli* was achieved according to [17]. Single-stranded DNA of derivatives of pUC 118 or 119 was prepared from *E. coli* JM 109 using M13K07 as helper phage [18]. DNA was sequenced with a Sanger dideoxy-mediated chain termintion method [19] using the Boehringer, Mannheim (FRG), sequencing kit with α-^{35}S-dATP (600 mCi/Mol; 10 mCi/ml) from Amersham as radioactive nucleotide and an LKB Makrophor electrophoresis apparatus. Sequence analysis was performed on an IBM 3090-400E computer using the BIO program (libraries: EMBL for DNA and Swiss Prot for proteins). Other methods used for molecular cloning were based on [20]. Hydropathy plots were constructed according to [21]. Hybridization with probes C and A was performed at 48°C and 52°C, respectively. Autoradiography of ^{32}P-active blots on Whatman-MM filter paper covered by Saran Wrap placed between two Cronex intensifying screens (DuPont) in a Cronex cassette (DuPont) was by overnight exposure to a Kodak x-ray film (Kodak x-omat) at room temperature. When ^{35}S was used exposure was for 2-3 days.

RESULTS AND DISCUSSION

Southern blotting

Chromosomal DNA digests of *Synechocystis* 6803 prepared with a variety of restriction endonucleases were sized on 0.4 % ultra-pure agarose gels and blotted onto nylon membranes. Hybridization with oligonucleotide probe C (radio-actively labeled with ^{32}P) resulted in the identification of only one band in each chromosomal digest while a similar hybridization with oligonucleotide probe A gave up to three hybridizing bands per fragment each of

which, when cloned and sequenced, did not show any aa similarity to a known coxII protein (results not shown). From this it is concluded that, while probe C indeed recognizes one and only one gene locus on the *Synechocystis* chromosome, viz. the Cu_B-binding site of coxI [22] which therefore must be assumed to be very similar in *P. denitrificans* and *Synechocystis* 6803, this does not seem to be the case for the usually invariant "aromatic" 7-aa stretch of *P. denitrificans* according to which probe A had been specifically designed [22] but which reads HQWYWSY in *P. denitrificans* but IQYAWIF in *Synechocystis* (also cf. Fig. 3).

Cloning of the ctaC (= coxII = coxB) gene

The 19-kb oligonucleotide probe A, originally designed according to the aromatic aa stretch in *P. denitrificans* COII [22], unexpectedly failed to detect a homologous gene locus in chromosomal DNA digests of *Synechocystis* 6803 [11]. However, probe C (directed towards the highly conserved Cu_B-binding region of the *P. denitrificans* COI protein [22] gave specific binding to a 2.2 kb PstI-KpnI DNA restriction fragment which, in addition to the probe C-binding gene locus, also contained the entire coding region of ctaB (Fig. 1). Thus it was possible, with the aid of an anti-ctaD (coxI) probe to clone and sequence the ctaB (coxII) gene of *Synechocystis* 6803 which encodes the COII protein, the most characteristic polypeptide subunit of all cyt c oxidases as it contains the cyt c-binding site and the redox-active, EPR-visilbe Cu_A [23-25].

DNA sequence and deduced aa sequence

Fig. 2 gives the complete nt and deduced aa sequence for the *Synechocystis* ctaC gene and COII protein, respectively. Fig. 3 shows alignments of

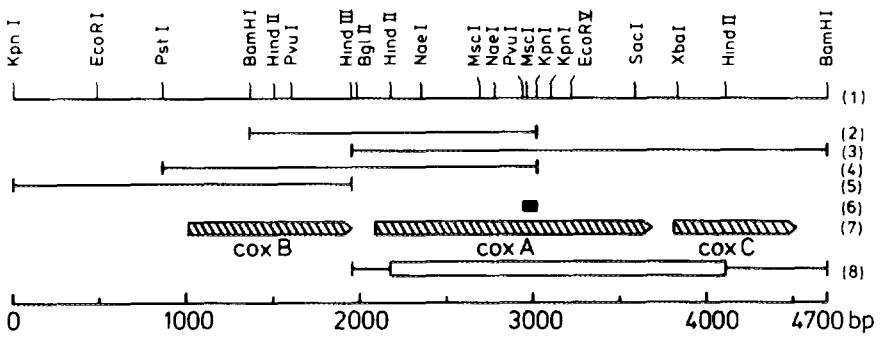

Fig. 1. Sequencing strategy of the cta (= cox) operon of *Synechocystis* PCC6803 encoding the cytochrome c oxidase (cytochrome aa_3). Starting from a probe C-binding gene locus on various chromosomal DNA restriction fragments subcloned into pUC 18, plasmid pDAUV 26 (line 4 in the figure) which contained both probe C-binding site and the entire coding region of coxB (= ctaC = coxII) the latter was cloned and sequenced. The organization of three cox genes in an operon as shown in the figure is highly suggestive (D. Alge and G. A. Peschek, unpublished). Lines 2, 3, 5 and 6 denote plasmid constructs not used for the present purposes. Line 7 illustrates the putative organization of the cta (cox) operon.

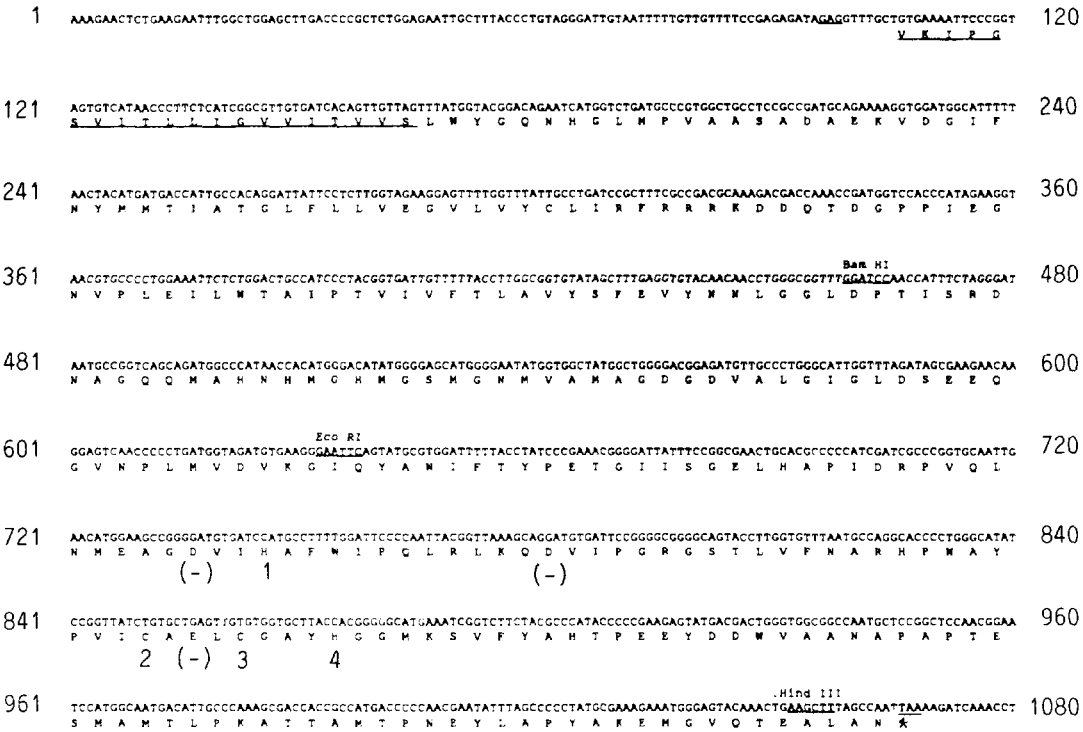

Fig. 2. Complete nt and deduced aa sequence of the ctaC (coxB) gene of *Synechocystis* PCC6803. Putative leader sequence and major restriction sites (cf. Fig. 1) are underlined, as are the putative start (GAG) and stop (TAA) codons. Numbers 1, 2, 3 and 4 below the letters mark putative Cu_A-binding aa. (-) marks putative cyt c-binding carboxylic acid residues. Note that "E183" (cf. Ref. 25) is replaced by a threonine (cf. Fig. 3).

deduced aa sequences of COII proteins from various cytochrome aa_3-containing eukaryotic and prokaryotic organisms including the cyanobacteria *Synechococcus vulcanus* [26] and *Synechocystis* PCC6803 (this paper). Also from hydropathy plots (Fig. 4) it is seen that not only the primary structure but also the secondary structure (protein fold) of *Synechocystis* COII is that of a typical cyt *c* oxidase subunit II protein. It has a leader sequence of 20 aa basically similar to other cytochrome aa_3-containing species. The calculated mol-%GC ratio of the protein is 48.3 (47.5 for the whole cta operon; not shown here) which is in good agreement with a value of 47.5 as published for the DNA from *Synechocystis* 6803 [27] giving the genome size of this species as 1.8×10^9 daltons [28].

However, a few minor features of the cyanobacterial COII sequence at variance with most other COII proteins (Fig. 3) may deserve closer attention: The typical stretch of aromatic aa (IQYAWIF), believed to act as an electron-conducting "wire" between ferrocytochrome c and the Cu_A [29] is shorter and more interrupted as compared to other COII proteins. Only three of the usually four invariant carboxylic acid residues thought to form the cyt c-

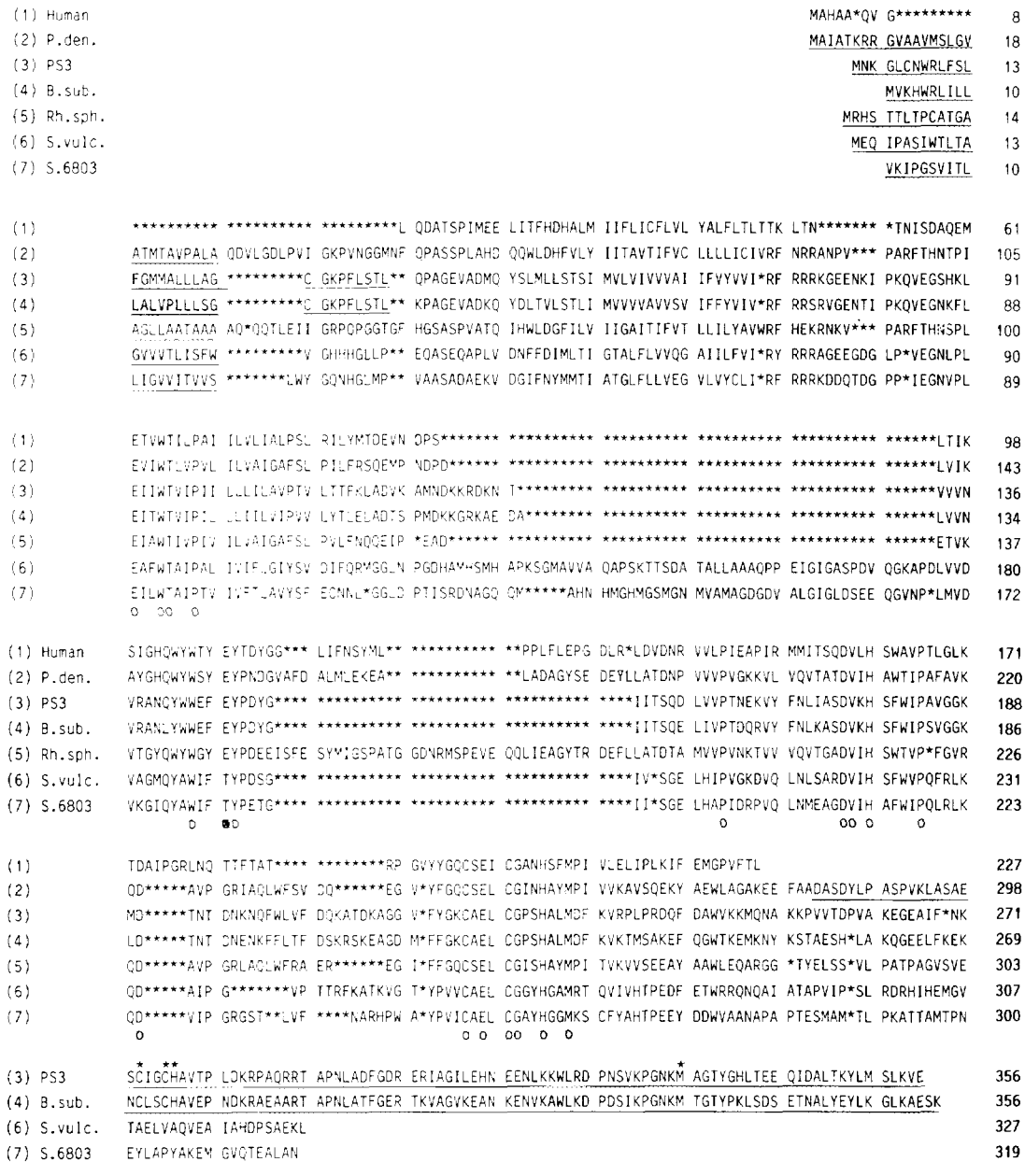

Fig. 3. Alignment of the deduced aa sequences of COII proteins from human mitochondrial cytochrome c oxidase [25], *Paracoccus denitrificans* [25], the Gram positive bacillus PS3 [33], *Bacillus subtilis* [32], *Rhodobacter sphaeroides* [40], *Synechococcus vulcanus* [26], and *Synechocystis* PCC6803 (this paper). Strictly conserved residues are marked with o below the letters. Partly putative leader sequences *and* C-terminal extensions are underlined. Note that these extensions are fused cyt c moieties in *B. subtilis* and PS3 (heme c-binding aa marked with * above letters) but cleaved off the *P. denitrificans* protein before incorporation into the membrane [25]. Also the putative Cu_A-binding aa and cyt c-binding carboxylic acid residues are marked with an asterisk, while the E183 which, in the cyanobacterial COII protein is replaced by threonine is marked with ⊗ (For the putative secondary structure of COII cf. Fig. 4).

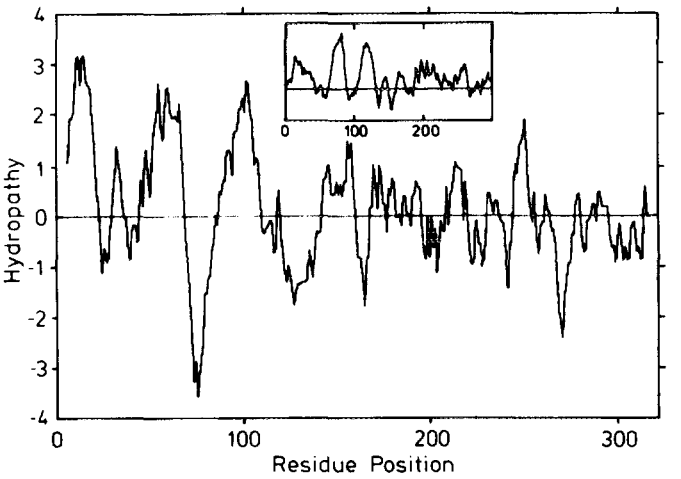

Fig. 4. Hydropathy plots of COII proteins from *Synechocystis* PCC6803 and *P. denitrificans* (inset). The amphipathic profiles were constructed according to deduced aa sequences (cf. Figs. 2 and 3) following the procedure of Kyte and Doolittle [21] and using a window length of 9 aa.

binding domain [30] are conserved in the *Synechocystis* COII protein, viz. D210, D225 and E250, while the position of the missing E183 is occupied by a threonine (Fig. 3; cf. Ref. 25) and a very similar situation obtaines in another cyanobacterium, *Synechococcus vulcanus* [26]. Contrary to this divergence, the putative Cu_A-binding histidines and cysteines are conserved also in the cyanobacterial COII proteins (Fig. 3). A further peculiar feature of cyanobacterial COII different from all other homologous proteins is the long (>50 aa) distance between the (periplasmic = intrathylakoidal) membrane surface and the cyt c-binding and cyt c-oxidizing domain (Fig. 3) which, together with the altered "aromatic" aa stretch (see above) might be responsible for the peculiar affinity of cytochrome oxidases from (unicellular) cyanobacteria towards acidic c-type cytochromes at elevated ionic strength [31]. A further characteristic feature of cyanobacterial COII proteins is that they lack the C-terminal aa extension (of about 100 residues) which, in more or less thermophilic and Gram positive bacilli [32, 33], but also in the Gram negative *Thermus thermophilus* [34] encodes a cytochrome c moiety. This lack of covalently linked cyt c is particularly interesting in case of the thermophilic *S. vulcanus* [26]. On the other hand, cyanobacterial COII does have a short C-terminal aa extension, yet without heme c-binding cysteines (Fig. 3), somehow similar to the (albeit even shorter) *P. denitrificans* extension which is not present in the mature protein [25]. Table 1 shows the identity matrix for COII proteins from eight different, eukaryotic and prokaryotic species. In view of the rather different overall mol-% GC values of *Synechococcus* spp. (48-71) and *Synechocystis* spp. (35-48) (cf. Ref. 27)

Table 1 Identity matrix for COII proteins from different sources. Alignments of the cytochrome *c* portion in the C-terminal region were not included. In some instances conservative amino acid exchanges [41] were added to the number of identical residues (numbers in brackets). Deduced aa sequences of COII proteins were taken from the literature as follows: Human, maize and *Paracoccus denitrificans* [25], *Rhodobacter sphaeroides* [40], PS3 [33], *Bacillus subtilis* [32], and *Synechococcus vulcanus* [26].

Source	Human	Maize	P.den.	Rh.sph.	PS3	B.sub.	S.vulc.	S.6803
Human	227							
Maize	202 (231)	259						
P.den.	69	60	298					
Rh.sph.	50 (93)	68 (112)	140 (189)	303				
PS3	43	52	58	39 (74)	271[1]			
B.sub.	36	38	57	132	166	269[2]		
S.vulc.	47 (88)	45 (82)	59 (92)	54 (94)	72 (123)	71 (109)		
S.6803	33 (73)	45 (85)	50 (91)	51 (94)	67 (115)	66 (109)	123 (178)	319

[1] 356 aa with C-terminal cyt *c*.
[2] 356 aa with C-terminal cyt *c*.

the up to 55 % identical aa residues in COII proteins from the two organisms are rather surprising.

CONCLUSIONS

As judged from the gene sequence presented in the previous section it is almost certain that the cyanobacterium *Synechocystis* PCC6803 synthesizes a typical COII protein which, in vivo, is part of an aa_3-type cytochrome c oxidase. Immunoblots of membrane proteins [1, 3] did show a specifically cross-reacting cytochrome c oxidase subunit II protein (COII), and from spectrophotometric measurements the occurrence of aa_3-type cytochrome oxidase in cyanobacteria was inferred still earlier [4, 5]. But only now, from the attempts to isolate and purify the cytochrome c oxidase of cyanobacteria [1, 8-10], and from immunological identification of the enzyme in membrane preparations from 25 different species of cyanobacteria [1, 2, 6, 7; G. A. Peschek et al., unpublished] it is clear that this highly diversified, ecologically most successful and evolutionarily most important group of

oxygenic phototrophic prokaryotes contains aa_3-type cytochrome c oxidase as their typical respiratory terminal oxidase. Due to the fact that around 3.2 billion years ago [35-37] the immediate ancestors of contemporary cyanobacteria were the first to introduce O_2 into a previously anaerobic biosphere and atmosphere, and due to the fact that they themselves inevitably must have been the first to "sense" that O_2, it is tempting to suggest that the cyanobacterial cytochrome oxidase represents the primordial enzyme [38].

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