FEBS Letters 347 (1994) 90–94

FEBS 14129

The 'Hinge' protein of cytochrome *c* reductase from potato lacks the acidic domain and has no cleavable presequence

Hans-Peter Braun[a], Lothar Jänsch[a], Volker Kruft[b], Udo K. Schmitz[a,*]

[a]*Institut für Genbiologische Forschung GmbH, Ihnestr. 63, D-14195 Berlin, Germany*
[b]*Applied Biosystems GmbH, Brunnenweg 13, D-64331 Weiterstadt, Germany*

Received 9 May 1994

Abstract

The 'Hinge' protein of cytochrome *c* reductase from fungi and mammals is thought to support electron transport from cytochrome c_1 to cytochrome *c* and was reported to be one of the most acidic proteins known. Isolation and analysis of cDNA clones of the first 'Hinge' protein from a plant source reveals that it has a surplus of basic residues in potato. While the overall identity between the deduced amino acid sequence of the potato 'Hinge' protein and the proteins from yeast and bovine is in the range of 40%, the characteristic acidic domain is lacking. Therefore the numerous theories on the function of the mitochondrial 'Hinge' protein seem not to apply for the protein from potato. Also the atypical acidic presequence of the 'Hinge' protein from fungi and mammals is absent as revealed by N-terminal sequencing of the isolated potato 'Hinge' protein. Functional implications of these results for the 'Hinge' proteins from other organisms are discussed.

Key words: 'Hinge' protein; Cytochrome *c* reductase; Respiratory chain; Protein import; Mitochondrion; *Solanum tuberosum*

1. Introduction

The mitochondrial cytochrome *c* reductase (EC 1.10.2.2.) contains 4–6 small subunits which are absent in the corresponding bc_1 complexes from prokaryotes (for reviews see [1,2]). One of them is the subunit VI (14.5 kDa) from yeast which is similar to subunit VIII (9.2 kDa) from bovine. This protein presumedly supports electron transfer from cytochrome c_1 to cytochrome *c* and was therefore proposed to be called the 'Hinge' protein [3,4]. The sequence of the 'Hinge' subunit is known for bovine [3], human [5] and yeast [6] and much interest has been focussed on this protein because it exhibits unique features in these organisms: (1) the 'Hinge' protein has an exceptionally high content of acidic amino acids (yeast, 45%; human, 33%; and bovine, 31%) culminating in a conserved domain with 8–25 consecutive glutamic or aspartic acid residues; (2) the 'Hinge' protein, which is nuclear-encoded and post-translationally transported into mitochondria is the only example having a highly acidic presequence that is cleaved off upon import into the organelle, while all other known mitochondrial presequences are strongly basic.

Although different biochemical and genetic approaches were applied to analyse the 'Hinge' protein its exact function is still unclear. Upon cleavage of cytochrome *c* reductase the 'Hinge' protein from bovine is part of a 'cytochrome c_1-subcomplex' together with cytochrome c_1 and a 7.2 kDa subunit [3,7,8]. The purified 'Hinge' protein from bovine was shown to be essential for complex formation between the isolated cytochromes *c* and c_1 [4,9,10] and to increase electron transfer between these two proteins at low ionic strength [11]. It can be crosslinked with both cytochromes and also with the 7.2 kDa subunit which was mentioned above [12,13]. Yeast cytochrome *c* reductase is still active after deletion of the gene encoding the 'Hinge' protein [14–18] but the activity of cytochrome *c* reductase decreases. There is some controversy about the dimension and the physiological significance of this decrease. Schopping and coworkers [14,16] observed a reduced bc_1 activity only at conditions of high ionic strengh whereas Schmitt and Trumpower [17] reported a 50% drop in activity at physiological conditions. Recently a deletion mutant for the yeast 'Hinge' protein was shown to cause temperature-sensitive petite growth [19]. The mutant is unable to generate mature cytochrome c_1 at non-permissive temperatures. Taken together there is a consensus that the 'Hinge' protein supports physical and physiological interaction between cytochrome *c* and cytochrome c_1 under certain conditions, but the reaction mechanism, the role of the acidic domain and the import pathway of the 'Hinge' protein are not understood. Here we report on the first molecular characterization of the 'Hinge' protein from a plant. The protein exhibits striking differences in comparison to the 'Hinge' proteins from other organisms.

2. Materials and methods

Copy-DNA clones for the potato 'Hinge' protein were isolated using a λgt11-library of potato tuber (*Solanum tuberosum*, var. 'Desirée') and a mixture of degenerative oligonucleotides which were derived from an internal amino acid sequence of the 'Hinge' protein (subunit 8) of cytochrome *c* reductase [20]. The mixture contained the full complement of sequences that could potentially encode the nonapeptide

*Corresponding author. Fax: (49) (30) 8300 0736.

SSDI 0014-5793(94)00515-W

Thr-Gly-Gln-Tyr-Phe-Asp-Tyr-Trp-His (384 combinations). End-labelling of the oligonucleotides was performed with T4 polynucleotide kinase by the use of [γ-^{32}P]dATP. Screening of the library, DNA cloning and DNA sequencing was carried out according to standard procedures [21]. Sequences were analysed on a VAX computer using the GCG-software package [22].

Cytochrome *c* reductase from potato was purified by affinity chromatography, ultrafiltration and gelfiltration as described previously [23]. The subunits of the isolated enzyme complex were separated by SDS-PAGE using the glycine or tricine buffer system [24,25] and either stained with Coomassie blue or blotted onto PVDF membranes. Transferred proteins were visualized with Ponceau-S and the 'Hinge' protein was cut out from the membranes for direct N-terminal amino acid sequence determination (outlined in [20]). For the production of antibodies against the potato 'Hinge' protein about 150 μg of the subunit were detached from the PVDF membranes by 80% formic acid, lyophilized and used for the immunization of one rabbit. The generated serum was testet against purified cytochrome *c* reductase from potato by immunoblotting as described before [26].

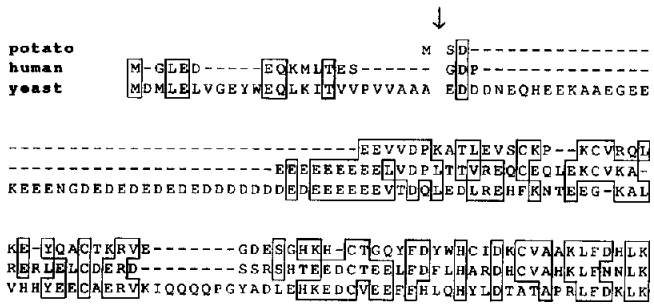

Fig. 2. Alignment of the potato 'Hinge' protein and the 'Hinge' proteins from human [5] and yeast [6,31]. Identical residues are boxed and dashes are introduced to maximize sequence similarity. The arrow indicates the cleavage site within the 'Hinge' precursor proteins from yeast and human (in potato only the N-terminal methionine is removed). The sequence from bovine [3] is not included in the alignment as it shows 94% identity to the sequence from human.

3. Results and discussion

Screening of the λgtll-library of potato tuber with a mixture of degenerative oligonucleotides led to the isolation of three cDNA clones for the potato 'Hinge' protein termed pCR12-1 – pCR12-3. The inserts of 0.5 kbp were analysed and revealed identical sequences (Fig. 1). They include an open reading frame (orf) of 207 bp which encodes a protein of 69 amino acids. The orf seems to code for the entire protein as there is an in-frame stop codon upstream of the start codon. The 3′ non-coding region comprises 201 bp, is followed by a polyA tail and includes a polyadenylation signal (AATAAA) 34 nucleotides upstream from the polyA tail. Comparison between the deduced amino acid sequence and the 'Hinge' proteins from other organisms reveals striking similarities. Based on the alignment in Fig. 2 the potato 'Hinge' protein shows 43% sequence identity with the corresponding protein from human and 35% with the protein from yeast (the identity between the sequences from human and yeast is 41%). The conservation of 'Hinge' proteins from very distantly related organisms is thereby in the same range as the conservation between the respiratory subunits and significantly higher than the one between other 'small' proteins of cytochrome *c* reductase from the same organisms [26–29]. The potato 'Hinge' protein has also some homology with a 19 kDa cysteine-rich subunit of the mitochondrial NADH-dehydrogenase from bovine ([30]; 27% identity in a strech of 50 amino acids, including four conserved cysteins) but does not show significant similarity with the calcium-binding site of calmodulin as previously reported for the 'Hinge' protein from yeast [31]. Since homology to calmodulin was also not verified with the sequence of the bovine 'Hinge' protein [1] its potential relevance most likely remains restricted to the 'Hinge' protein from yeast.

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CTAGAACAGTCAGGTGATTTTGCAGAATCGAAGTCTTCGTTTCCAATATCTCTTTGCC    58

 M   S   D   E   E   V   V   D   P   K   A   T   L   E   V     15
ATG TCG GAC GAG GAA GTT GTT GAC CCA AAG GCA ACA CTG GAA GTA    103

 S   C   K   P   K   C   V   R   Q   L   K   E   Y   Q   A     30
AGT TGC AAA CCT AAG TGT GTA AGG CAA CTA AAG GAG TAT CAG GCA    148

 C   T   K   R   V   E   G   D   E   S   G   H   K   H   C     45
TGT ACT AAA AGG GTA GAA GGT GAT GAA TCA GGG CAC AAA CAT TGC    193

 T   G   Q   Y   F   D   Y   W   H   C   I   D   K   C   V     60
ACT GGA CAG TAT TTT GAT TAT TGG CAC TGC ATT GAC AAA TGT GTT    238

 A   A   K   L   F   D   H   L   K   *                        69
GCT GCG AAG TTA TTT GAC CAC CTC AAG TAA CAAGGATATAAGTTGTTGA    287

TCCCTTGCAATTTATCTGTTTTTTTGTTGTTGAACAAGTCATTACCATATTATTCCTCA    346

CTGTGCTGCTGACTTGCAACCCTTTCAATCAACTTGGTTGCTGCATGGAAAATTTTGAA    405

CTATGCACATCTTAAAAAAATGATTAATAAATTATACTCGTGGGTTAAATTTTTTGAAC    464

CCTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                             498
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the insert of clone pCR12-2. The putative polyadenylation site is underlined.

The potato 'Hinge' protein has 40% charged residues (yeast: 60%, human: 51%). Most notable is the virtually complete absence of the characteristic acidic domain comprising 25 consecutive acidic amino acids in yeast and 9 in human. Consequently the overall amino acid composition of the potato 'Hinge' protein is very different from the one of other organisms as it has more basic than acidic residues (potato: +3; human: −12; yeast: −37). As a result the isoelectric point of the 'Hinge' protein from potato is dramatically changed (yeast: 3.8, human: 4.2, potato: 6.9). Another short stretch that is absent in the 'Hinge' protein from potato contains four successive glutamine residues and seems to be unique to yeast (Fig. 2). The secondary structure of the potato 'Hinge' protein is predicted to be mainly α-helical and the protein does not contain an area of uncharged/hydrophobic amino acids that is large enough to span a biological membrane. Therefore it is most likely anchored in the cytochrome *c* reductase by interaction with other subunits of the complex. The 'Hinge' protein from potato contains six cysteines whereas the three other

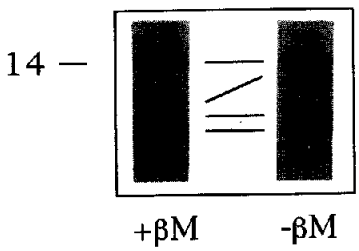

Fig. 3. SDS-PAGE of isolated cytochrome *c* reductase from potato in the presence or absence of β-mercaptoethanol (+βM/−βM). The gel was stained with Coomassie blue. Only the four low molecular weight subunits of the protein complex are shown (the size of a 14 kDa standard protein is indicated on the left). The 'Hinge' protein (second largest band) exhibits a significant shift if electrophoresed under the two different conditions.

small subunits of cytochrome *c* reductase from potato lack this amino acid (Braun and Schmitz, unpublished). The presence of β-mercaptoethanol during SDS-PAGE of isolated cytochrome *c* reductase specifically increase the electrophoretic mobility of the 'Hinge' subunit (Fig. 3) indicating the occurence of disulfide bridges in the mature protein. Five of the six cysteines of the potato 'Hinge' protein are conserved in bovine, four of which were previously shown to form two disulfide bridges [32]. The corresponding bridges would link Cys-17 with Cys-59 of the potato sequence and Cys-31 with Cys-45. Theoretically a third disulfide bridge could be formed in the protein from potato (between Cys-21 and Cys-55; both residues have a distance of four amino acids to the bridge forming cysteines at positions 17 and 59). Interestingly the number of cysteine residues of the 'Hinge' protein from different organisms (potato, 6; bovine and human, 5; yeast, 2) is inversely proportional to the excess of acidic residues raising the possibility that a tighter folding of the protein by disulfide bridges might lead to an efficient exposure of a reduced number of acidic amino acids.

The calculated molecular weight of the potato 'Hinge' protein as deduced from the open reading frame of the corresponding clones is 7971 Da. If analysed by SDS-PAGE in 'Hinge' protein of cytochrome *c* reductase from potato (subunit 8) was reported to have a molecular weight of 9–12 kDa [23,33]. However, the apparent sizes of the 'Hinge' proteins from yeast and bovine were overestimated [3,6]. Reinvestigation of the apparent molecular size of the potato 'Hinge' protein with the SDS-PAGE system outlined by Schägger and von Jagow [25], which is especially suitable for the separation and size determination of small proteins revealed a molecular weight of only 8 kDa (Jänsch and Braun, unpublished). Consequently the calculated and the apparent molecular size of the 'Hinge' protein from potato is identical and the protein seems to lack a mitochondrial targeting sequence. To prove this hypothesis the 'Hinge' subunit of cytochrome *c* reductase from potato ws subjected to direct protein sequencing by cyclic Edman degradation. The obtained sequence was SDEEVVDPKATLEVSXKP (Fig. 4). In contrast to the 'Hinge' proteins from fungi and mammals, which have an acidic presequence [5,6] that is removed upon import into the mitochondrion [34], only the initiator methionine is absent from the mature potato 'Hinge' protein.

There is an increasing number of nuclear encoded mitochondrial proteins which are known to be imported without a cleavable presequence, including some of the other low molecular weight subunits from cytochrome *c* reductase [1]. For several mitochondrial proteins a presequence is obviously dispensable as the absence of cleavable targeting information is a well conserved feature of these proteins in the organisms analysed. Only in very few cases the position of the targeting information varies in homologous proteins from different organisms. Examples are the ADP/ATP translocator, which has a presequence only in plants [35,36] and cytochrome c_1, that lacks a presequence in trypanosomes [37]. Also the 'Hinge' protein belongs to these rare exceptions.

There has been much speculation on the acidic presequence of the 'Hinge' protein from yeast and mammals and on the question whether it encodes targeting information or rather represents a structural determinant which is post-translationally removed in order to lock the protein into its native conformation [31]. Typical sequences for mitochondrial targeting show a preponderance of basic and hydroxylated amino acids and an

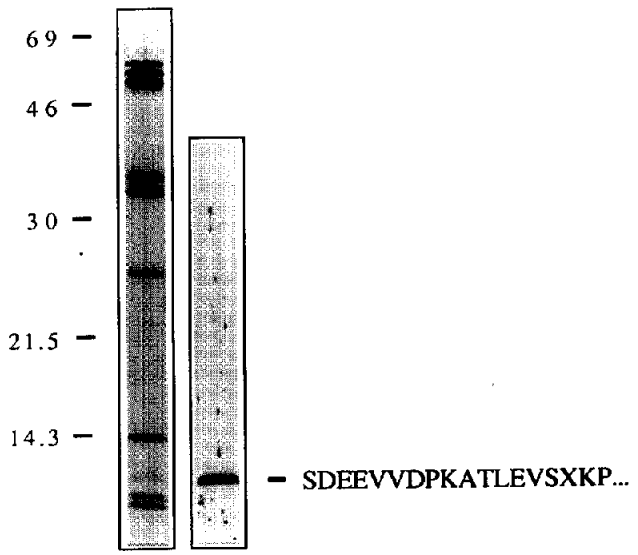

Fig. 4. Specificity of an antiserum which was generated against the potato 'Hinge' protein. Purified cytochrome *c* reductase was stained with Coomassie blue (left) or blotted onto nitrocellulose and incubated with the antibody directed against the 'Hinge' ıbunit (right). The numbers on the left indicate the size of standard proteins (in kDa). The N-terminal sequence of the mature protein was determined by cyclic Edman degradation and is given on the right of the blot. (As reported for other organisms the 'Hinge' protein has poor colour values after staining with Coomassie [4]).

amphiphilic α-helical secodary structure [38]. As neither the presequence nor internal stretches of the 'Hinge' proteins from yeast and mammals have these characteristic feature an unique import pathway including a specific import receptor was postulated [5,31]. The absence of a presequence of the 'Hinge' protein from potato can be interpreted either as an argument for thus far unidentified internal targeting information of 'Hinge' proteins in general or as an unique adaptation for the import of a 'Hinge' protein that lacks the acidic domain. Antibodies which specifically recognize the 'Hinge' protein from potato have been generated (Fig. 4) to analyse its import into isolated mitochondria and to perform topological studies.

So far most theories on the topology and function of the 'Hinge' protein concentrate on the presence of the stretch of acidic amino acids in the proteins from fungi and mammals. According to a wide-spread hypothesis this domain is responsible for the binding of cytochrome *c* [4,6]. Cytochrome *c* contains clusters of basic amino acids [39] and has an isoelectric point above 10. However, although the occurrence of a physical interaction between the 'Hinge' protein and cytochrome *c* was proven in biochemical and genetic experiments [4,11,18], an ionic attraction between these two components is up to now only hypothetical and most probably not valid for potato. Interestingly cytochrome *c* from potato has a reduced number of basic amino acids if compared to cytochrome *c* from mammals and fungi (potato, 17; yeast and bovine, 23 basic residues; [40–42]). On the other hand potato cytochrome *c* reductase efficiently binds cytochrome *c* from mammals as it can be purified by affinity chromatography with immobilized horse cytochrome *c* [23] and as it has high turnover numbers with horse cytochrome *c* as a substrate (170 s^{-1}, [33]). It is therefore possible that the acidic domain of the 'Hinge' protein from fungi and mammals is not a prerequisite for the binding of cytochrome *c*. The cytochrome c_1 subunit of cytochrome *c* reductase itself has two acidic domains which are conserved in all organisms analysed so far and which were shown to be involved in the binding of cytochrome *c* [12,43]. Alternative ideas on the mechanism of interaction between the 'Hinge' protein and the cytochromes *c* and c_1 should be taken under consideration.

The absence of the surplus of acidic residues in the potato 'Hinge' protein also sheds new light on other theories concerning the functional role of this subunit. It was proposed that the clustering of acidic amino acids is likely to change the p*K*s of individual residues, causing a gradient within the acidic area which might function as a 'proton sink' [6]. Another hypothesis on the 'Hinge' protein predicts a pH-dependent change in the secondary structure of the cluster of acidic amino acids which could regulate the activity of the 'Hinge' protein [6,17]. Physiological studies with the potato 'Hinge' protein may allow a critical reexamination of these theories.

Acknowledgements: We wish to thank H. Mentzel for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, Grant Schm. 698/2.

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