Bovine Coupling Factor 6, with Just 14.5% Shared Identity, Replaces Subunit h in the Yeast ATP Synthase*

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The mammalian mitochondrial ATP synthase is composed of at least 16 polypeptides. With the exception of coupling factor Fₐ, there are likely yeast homologs of each of these polypeptides. There are no obvious sequence homologs of Fₐ, as predicted from primary structure comparisons of the yeast enzyme at 3.9 Å resolution revealed the structure of the enzyme; the disruption of any of the corresponding structural genes leads to a lack of assembly of the holo-complex (12). Recently, the establishment of the structure of the yeast enzyme at 3.9 Å resolution revealed the structure of the F₁ and the subunit c oligomer of F₁ (13). Among the supernumerary subunits of the yeast ATP synthase Fₐ, subunit h has been described as an essential component because inactivation of the ATP₁₄ gene led to a lack of oxidative phosphorylations (14). Recently cross-linking experiments (15) have positioned this hydrophilic and acidic component of 10,408 Da close to subunit 4 (subunit b), a component of the second stalk of the ATP synthase. In this paper we report the complementation of a yeast strain devoid of the yeast ATP synthase subunit h by a single copy vector bearing a DNA sequence encoding the bovine coupling factor 6. This is a rather remarkable result because subunit h and bovine Fₐ share only 14.5% sequence identity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Nucleic Acid Techniques—The S. cerevisiae strain D273-10B/AYU (MATa, met6, ura3, his3) (16) was the wild type strain. The strain with the null mutation in ATP₁₄ (MATa, met6, ura3, his3, ATP₁₄:URA3) has been described (14). The ΔATP₁₄ strain containing the plasmid pBF₆ was obtained by transformation of the yeast ΔATP₁₄ gene by the nonintegrative single copy vector, pRS313, which contains the coding region of mature bF₆, the leader peptide of the β-subunit of the yeast ATP synthase, and the upstream and downstream transcriptional controlling elements of the ATP₂ gene.

The expression plasmid for expression of bF₆, pBF₆, was made essentially as described (17). In this scheme, the coding region of mature bF₆ replaces the coding region of the gene encoding the β-subunit of the ATP synthase, ATP₂. This allows the expression of bF₆ to be under the same controls as that of the ATP₂ gene. The coding region of bF₆ is amplified by PCR, this PCR fragment is used to directly replace the coding region of the ATP₂ gene. The replacement occurs by site-specific homologous recombination effected in yeast. In addition to the bases required to direct the synthesis of the coding region of bF₆, the PCR

1 The abbreviations used are: bF₆, bovine coupling factor 6; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DSP, dithiobis(succinimidylpropionate); OSCP, oligomycin-sensitivity-conferring protein; PCR, polymerase chain reaction.
primes also contain a 30-base sequence that targets the PCR product to the desired site of recombination on the AT2P2 gene, in this case at the end of the region coding for the leader peptide and at the 3′-untranslated part of the gene. Recombination is effected in vivo in yeast and occurs across a linearized plasmid (pRS314) (18) that contains the AT2P2 gene that is cut in the center (gap repair) (for a diagram of this method, see Ref. 17). The recombination event occurs at the region encoding the leader peptide, at one end, and at the stop codon of the coding frame of BF (a clone containing the cDNA for bovine BF was kindly provided by Dr. John E. Walker, Cambridge, UK). In this manner, the coding region of mature BF replaces the coding region of the AT2P2 gene.

The DNA sequence of the chimeric gene was sequenced to ensure the correct recombination event and to ensure the absence of any mutations. DNA sequence analysis was performed at the Iowa State University sequencing facility (Ames, Iowa). The chimeric gene was removed from the plasmid by digestion with XbaI and SalI and subcloned into the low copy vector pRS313 at the same restriction sites (18).

The forward and reverse primers used in the PCR reaction were: bx F2-pr: CTT CTA GCC ACT TCG TGG AAA AGA TGT AGT GCC TCA aat gag gtt ctt gct gtg and bx F2-re-pr: CTT CCC TGG GTT TAA GCT TTA TTT CTT CTA gaa tgt ttt ctc gcg, respectively. The lowercase letters correspond to the region that primes DNA synthesis in the PCR reaction using the cDNA of BF as the template. The capital letters correspond to the target site for homologous recombination in the plasmid containing the gene encoding the β-subunit of the F2-ATPase (ATP2). The underlined sequence corresponds to the additional Ala-Ser codons. These were added because it is frequently the case that Ala-Ser are the first two amino acids after the processing site of the leader peptide. The Ala-Ser are thus positioned after and adjacent to the leader sequence of what corresponds to the leader peptide of the β-subunit of the ATPase.

Biochemical Procedures—Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as carbon source (19) and harvested in logarithmic growth phase. Mitochondria were prepared according to (20), frozen as droplets in liquid nitrogen, and stored at −70 °C. Protein amounts were determined according to Lowry et al. (21) in the presence of 5% SDS using bovine serum albumin as a standard. Oxygen consumption rates were measured with NADH as substrate (22). Variations of transmembrane potential (∆Ψ) were evaluated by measurement of fluorescence quenching of rhodamine 123 with an SFM Kontron fluorescence spectrophotometer (23). The specific ATPase activity was measured at pH 8.4 according to Somlo (24) and modified as follows. Freshly prepared mitochondria were diluted with the same volume of either the isolation buffer (0.6 M mannitol, 2 mM EGTA, 10 mM Tris-maleate, pH 6.8) or 0.75% Triton X-100 (w/v). Aliquots were used for protein concentration measurement, and determination of ATPase activity was done as follows: mitochondrial protein (50 μg) was incubated for 2 min in reaction medium (0.9 ml, 0.2 M KCl, 3 mM MgCl2, 10 mM Tris-HCL, pH 8.4) in the presence or absence of P2 inhibitors. The reaction was started with the addition of 5 mM ATP and stopped after 2 min by the addition of 0.3 M trichloroacetic acid.

Mitochondrial Triton X-100 extracts were sedimented on sucrose gradients as follows. Mitochondrial protein (3 mg at 10 mg/ml) was incubated with an equal volume of 0.75% (w/v) Triton X-100 (w/v). The extract was spun at 100,000 × g for 10 min at 4 °C. The supernatant was loaded on the top of a 10–30% linear sucrose gradient containing 0.1% Triton X-100, 1 mM ATP, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0. The gradient was centrifuged at 4 °C for 17 h, 46 min in a SW41 rotor (Beckman) at 41,000 rpm, and fractions (1 ml) were collected and used for analysis.

Cross-linking Experiments and Analyses—Frozen mitochondria isolated from wild type and mutant cells were thawed, washed twice with 0.6 M mannitol, 2 mM EGTA, 50 mM triethanolamine-HCl, pH 8.0, and suspended in the same buffer at a protein concentration of 10 mg/ml. Samples were incubated for 20 min at 30 °C in the presence or absence of DSP dissolved in dimethylformamide, and the reaction was quenched by the addition of 10 mM Tris, pH 8.0. The ATP synthase was extracted with 0.375% (w/v) Triton X-100, and after incubation for 15 min at 4 °C, the extract was spun at 100,000 × g for 15 min at 4 °C, and the supernatant was used for analysis.

Bone F6 was released from the ATP synthase (free BF) by sonication as follows. Washed mitochondria from yeast, ΔATP14 + BF2, were suspended in 100 mM NaCl, 22 mM triethanolamine, pH 8.0, at a final concentration of 10 mg protein/ml and sonicated four times for 30 s at 120 V (Annemasse sonicator). The sample was centrifuged at 435,000 × g for 1 h at 4 °C. Aliquots of the supernatant containing free BF2 were incubated with DSP and analyzed either by Western blot or loaded on a 10–30% linear sucrose gradient, as described above.

SDS-polyacrylamide gel electrophoresis was according to Schägger and Von Jagow (25). Nicotinucleoside membranes (Membrane Protein BA83, 0.2 μm from Schleicher & Schuell) were used for Western blot analyses. The polyclonal antibodies, anti-BF2, were kindly provided by Dr. Yousaf Hatemi (Scripps Research Institute, La Jolla, CA) and used with a dilution of 1:7,500. Antibodies against subunit 4 and the α-subunit were used with dilutions of 1:10,000 and 1:100,000, respectively. Mitochondria were incubated with peroxidase-labeled antibodies and visualized with the ECL reagent of Amersham Pharmacia Biotech. Molecular mass markers (Benchmark Prestained Protein Ladder) were from Life Technologies, Inc. Sequence alignments were performed as in (26). Secondary structure predictions were made with the program at the PredictProtein Server.

RESULTS

The Coding Sequence of the Bovine Coupling Factor 6 Complements a Null Mutant Strain Devoid of Subunit b—For expression in yeast, the cDNA encoding mature BF was fused to the leader sequence of the yeast β-subunit. In addition, the codons for Ala-Ser were added to the front end of the codons encoding the mature BF as described (17). The expectation is that leader sequence will be cleaved in front of the Ala-Ser residue after import into the mitochondrion providing an N-terminal sequence of ASNKELD . . .

The strain containing the null mutation in the ATP14 gene (14), which encodes subunit h, was transformed by a single copy vector (pRS313) bearing the selection marker HIS3 and the DNA encoding the bovine BF, pbF2. The transformants were tested for growth on a complete medium containing glycerol as the sole carbon source, YPG (3% glycerol, 2% peptone, 1% yeast extract). Growth on medium containing glycerol or lactate indicates that the cells are able to use ATP via oxidative phosphorylation and thus have a functional ATP synthase. All of the transformants were able to grow on YPG at 28 °C but not at 37 °C (Fig. 1). One of the transformants was selected, named ΔATP14 + BF2, and this strain had a generation time of 90 min as compared with 164 min for the wild type strain, at 28 °C in liquid medium containing lactate as the carbon source. Loss of the plasmid, by growing the cells on a complete medium with glucose as carbon source, resulted in a concomitant loss in the ability to grow on YPG or ATP14 medium devoid of histidine and on YPG medium (not shown). These results indicate a functional homology between yeast subunit h and BF2.

Phenotypic Analyses of the Complemented Yeast Strain—Mitochondria were prepared from ΔATP14 + BF2 strain to examine the effectiveness of BF2 in replacing subunit h in the structure and function of the ATP synthase. Respiration rates

FIG. 1. Complementation of the null mutant ATP14 by a single copy vector bearing the bovine BF gene. Wild type (wt) strain (D273–10B/A/H/U), ΔATP14 null mutant strain, and the complemented strain ΔATP14 + BF2 were serially diluted, and 3 μl of each dilution corresponding to the same cell number were spotted on solid complete medium containing glycerol as the carbon source (YPG). The cells were grown for 120 h at either 28 or 37 °C, as indicated.
were measured with NADH as substrate (Table I). In the presence of CCCP, the uncoupled respiration rates of mitochondria isolated from the wild type and ΔATP14 + bF6 strains were similar. The main difference between the two preparations is the respiration rate associated with the phosphorylation of ADP (State 3) where the respiration rate of ΔATP14 + bF6 mitochondria is only 51% of the uncoupled respiration rate, as compared with 63% for the wild type. As a result, the respiratory control and the ATP/O ratio of ΔATP14 + bF6 mitochondria are lower than those of wild type mitochondria, thus reflecting a lower ATP synthase activity and a lower efficiency of oxidative phosphorylation.

At pH 8.4, the ATPase activity of ΔATP14 + bF6 mitochondria is partially inhibited by DCCD but not by oligomycin, both of which are inhibitors of F0 (Table II). Addition of Triton X-100 (3.75%) solubilizes the ATP synthase and increases the ATPase activity of wild type mitochondria by removing F1, the F1 inhibitor protein. This stimulation also occurred for the ΔATP14 + bF6 mitochondrial extract, but the sensitivity to DCCD was nearly abolished. These data suggest that the ATP synthase isolated from ΔATP14 + bF6 mitochondria was highly unstable, consistent with the temperature-sensitive phenotype of the cells on YPG medium (Fig. 1).

Potential (∆ψ) measurements were performed in the respiration medium (Fig. 2). With ethanol as a substrate, the addition of ADP promoted a transient decrease in the fluorescent quenching of rhodamine 123 because of proton uptake through F0 during ATP synthesis. This effect was less pronounced with mitochondria isolated from ΔATP14 + bF6 consistent with the low rate of state 3 respiration. The reversibility of the ATP synthase was also examined. Ethanol addition promoted a strong fluorescence quenching of rhodamine 123 because of the respiratory chain activity, and this was reversed by potassium cyanide. Finally, addition of ATP caused a fluorescent quenching because of its hydrolysis and the coupled pumping of protons out of the mitochondrion. Clearly, the ∆ψ generated by ATP hydrolysis in mitochondria isolated from ΔATP14 + bF6 is sensitive to either oligomycin or DCCD at concentrations used to inhibit the wild type mitochondria, but the intensity of the fluorescent quenching was not as large, and it was not as stable as compared with the wild type sample. These results suggest that the ATP synthase from ΔATP14 + bF6 is uncoupling during the course of the assay. However, the addition of oligomycin or DCCD did not affect the transmembrane potential generated by the respiratory chain, suggesting that passive proton conduction through F0 is not occurring in the absence of either ADP or ATP.

**Bovine F6 Associates with the Yeast ATP Synthase**—The prior genetic and biochemical studies indicate that bF6 can correct for the loss of subunit h, albeit not to wild type levels. If bF6 is acting directly by substituting for subunit h in the ATP synthase, bF6 should be associated with the ATP synthase devoid of subunit h. A number of biochemical studies were performed to test this hypothesis. First Western blot analysis using anti-bF6 antiserum detected a band with a relative molecular mass of 10.6 kDa that was present in the SDS-dissociated ΔATP14 + bF6, but not wild type, mitochondrial sample (Fig. 3A). The mature bF6 has a calculated molecular mass of 9,116 Da assuming that processing occurs just prior to the Ala-Ser residues. If the protein is not processed, other than removing the initiating Met, then it would have a mass of 12,653 Da. These results suggest the 10.6-kDa peptide represents bF6 and is likely processed at the predicted point. However, N-terminal sequence analysis has not been done on the yeast-expressed bF6 to verify the site of processing.

Association of bF6 to the yeast ATP synthase missing subunit h was determined by immunoprecipitation of the detergent (Triton X-100)-solubilized ATP synthase complex using antibodies directed against the α-subunit. However, unlike ATP synthase from wild type yeast, the antibodies precipitated only the F1 sector (not shown). This suggested that the association of F1 and F0 sectors was not stable during immunoprecipitation. This is consistent with the prior studies that indicated that the ATP synthase with bF6 was not as stable as the wild type enzyme. To capture the interactions between bF6 and components of the ATP synthase, the mitochondrial proteins were treated with the thiol-cleavable homobifunctional cross-linking reagent, DSP. Fig. 3B shows that the concentration of bF6 was greatly decreased upon incubation of mitochondria with 0.2 and 0.5 mM DSP, concurrent with the presence of bands of high molecular masses, as revealed by Western blot analysis. The most intense band displayed a relative molecular mass of 36 kDa that was present in the SDS-dissociated ΔATP14 + bF6, but not wild type, mitochondrial sample (Fig. 3A). The mature bF6 has a calculated molecular mass of 9,116 Da assuming that processing occurs just prior to the Ala-Ser residues. If the protein is not processed, other than removing the initiating Met, then it would have a mass of 12,653 Da. These results suggest the 10.6-kDa peptide represents bF6 and is likely processed at the predicted point. However, N-terminal sequence analysis has not been done on the yeast-expressed bF6 to verify the site of processing.

**TABLE I**

Oxidative phosphorylation measurements of isolated mitochondria

| Mitochondria | Respiratory rates | ATP/O | 
|--------------|------------------|-------|
|              | CCCP             | State 4|
| Wild type    | 1199 ± 42        | 302 ± 39|
| ΔATP14 + bF6 | 1090 ± 32        | 314 ± 41|
|              | 252 ± 20         | 561 ± 2 |
| Control      | 1.09 ± 0.13      | 0.80 ± 0.03 |

**TABLE II**

ATPase activities of isolated mitochondria

| Strains       | Control + Oligomycin + DCCD | Wild type | Tritic X-100 | ΔATP14 + bF6 | ΔATP14 + bF6 + |
|---------------|-----------------------------|-----------|--------------|--------------|---------------|
| Wild type     | 2696 ± 98                   | 243 ± 105 | 285 ± 12     | 669 ± 40     | 669 ± 34      |
| Tritic X-100  | 6749 ± 190                  | 3154 ± 57 | 1487 ± 41    | 6346 ± 59    | 6346 ± 59    |

Data are from typical experiments. Measurements were performed four times. Respiration rates were obtained with NADH as substrate. CCCP concentration was 3 μM.
Fig. 2. Mitochondrial energization monitored by fluorescent quenching of rhodamine 123. D273–10B/A/H/U mitochondria (wild type) and ΔATP14 + bF6 mitochondria were incubated in 2 ml of respiration medium. Additions were 0.6 mg of mitochondrial protein (m), 10 μl of ethanol (e), 37 μM ADP, 3 μM CCCP (c), 200 μM KCN (k), 1 mM ATP, 6 μg of DCCD (d), and 6 μg of oligomycin (o). Dotted line, additions were mitochondria, ethanol, KCN, ATP, and oligomycin. Dashed line, additions were mitochondria, ethanol, oligomycin, and DCCD.

Fig. 3. Cross-linking bF6 to putative components of the ATP synthase. A, wild type (wt) and ΔATP14 + bF6 mitochondria were dissociated and analyzed by Western blot. Blots were probed with polyclonal antibodies raised against subunits 1 and bF6. B, ΔATP14 + bF6 mitochondria (30 μg of protein) was cross-linked with 0.2 and 0.5 mM DSP and treated with 2-mercaptoethanol, as indicated, and the blot was probed with antibodies raised against bF6. (C) bF6 was released from F1F0 by sonication (see “Experimental Procedures”) and treated with DSP and 2-mercaptoethanol, as indicated, and the blot was probed with polyclonal antibodies raised against bF6.

To determine whether bF6 was cross-linked to the ATP synthase, mitochondria treated with DSP were extracted with Triton X-100, and the solubilized proteins were separated by sucrose-gradient centrifugation. Eleven fractions were collected and analyzed by Western blot. The samples were reduced before SDS-polyacrylamide gel electrophoresis to allow the clear identification of bF6. The blots were probed with antibodies raised against the α-subunit (an F1 subunit), subunit 4 (an F0 subunit), and bF6. The wild type ATP synthase sediments to fractions 4–8 as shown by the cosedimentation of the α-subunit and subunit 4 in the sucrose gradient (Fig. 4A). For the ATP synthase from mitochondria isolated from yeast ΔATP14 + bF6, the α-subunit and subunit 4 were distributed in a much broader range of fractions, suggesting both a more complex mixture of species and a less stable assembly of the ATP synthase. This occurred in the absence of reaction with DSP (not shown) or in the presence of 0.2 mM DSP (Fig. 4B). However, when the concentration of DSP was increased to 0.5 mM, then a significant fraction of bF6 was seen to sediment with the other subunits of the ATP synthase (Fig. 4C). This new location of bF6 in the sucrose gradient was not the result of nonspecific cross-linkings of bF6 to other proteins, because reaction of DSP with bF6, which has been freed and separated from F1F0, stayed at the top of the gradient, the same position of bF6 without reaction with DSP (Fig. 4, D and E). In conclusion, the results support the hypothesis that bF6 associates to the yeast ATP synthase.

DSP Primarily Cross-links Bovine F6 to Subunit 4—If bF6 was acting directly by replacing subunit 6, then not only should it associate with the ATP synthase, but it should also interact with the same peptides of the ATP synthase as subunit 6. To test this hypothesis, the protein partners of bF6 were determined by Western blot analysis after cross-linking the peptides with DSP. The results from Fig. 3 indicate that the major cross-linked species with bF6 had a molecular mass of about 36 kDa, suggesting that bF6 was cross-linked to a peptide 20–30 kDa in size. As a consequence, antibodies raised against subunits of the ATP synthase whose molecular mass were within this range, OSCP and subunits 6, d, and 4, were used in the analysis. In addition, long polyacrylamide slab gels were used to clearly identify the cross-linked products (Fig. 5).

In a wild type context, numerous cross-linked products involving subunit 4 have been reported, and the most intense band that was found in the 36-kDa region has been identified as 4+g (15). This same cross-linked product was present for both the ATP synthase from the wild type and ΔATP14 + bF6 strains (Fig. 5, A and B). However, there was an additional band at about 36 kDa that was obtained upon incubation with 0.2 and 0.5 mM DSP and that was seen with antibodies against either subunit 4 or bF6. This band ran slightly ahead of the 4+g cross-linked product, and it was absent from the wild type...
sample (Fig. 5B). This band is thus concluded to be a cross-linked product of bF6 and subunit d.

Two other bands of low intensity and showing relative molecular masses of 42 and 56 kDa could be heterooligomers containing at least bF6 and subunit d because the latter cross-linked products were absent from the wild type sample (Fig. 5C). Antibodies raised against subunit 6 and OSCP did not identify any bands that were also detected by antibodies against bF6 (not shown). The increased intensity of a 26-kDa band upon cross-linking of the ΔATP14 + bF6 mitochondria represents a cross-link involving subunit d and a small component of the yeast ATP synthase (Fig. 5C). The latter band was less apparent in the wild type sample. Thus, these results indicated that bF6 associated with subunit d of the ATP synthase consistent with the interactions of subunit h in the ATP synthase.

**Discussion**

Subunit h is an essential component of the yeast ATP synthase. It has been described as a supernumerary protein that, until this study, was not apparently related to any subunit described in other ATP synthases (14). Primary structural analysis of nucleotide data banks have identified the existence of an open reading frame in Schizosaccharomyces pombe (TrEMBL accession number 059673) that encodes a hypothetical protein 27% identical to subunit h and another in Botrytis fuckeliana (GenBankTM accession number AL115386) that also encodes a hypothetical protein 38% identical to subunit h. Yeast subunit h is essential for yeast to grow on a nonfermentable carbon sources, and mitochondria isolated from a yeast strain with a null mutation in subunit h have an ATPase activity that is oligomycin-insensitive and the catalytic sector dissociated from the membrane components (14).

Mammalian coupling factor F6 is an essential component of the mammalian mitochondrial ATP synthase. F6 is known to be required for restoration of ATP-F6 exchange and oligomycin-sensitive ATPase activity to factor 6-depleted ATP synthase (27, 28). It is also involved in the binding of F1 to F6 (29) and shields F6 from limited proteolysis (30). As such, F6 is thought to be required for the coupling of proton translocation to the synthesis of ATP.

The results in this manuscript are quite surprising and have important implications. Both genetic and biochemical data indicate that subunit h of the yeast ATP synthase is the homolog of mammalian coupling factor F6. This is a rather surprising because both of these subunits are essential components of their respective multimeric peptide complexes. Despite the apparent functional homology of subunit h with F6, primary se-
quence alignment of both subunits shows a very low sequence identity of just 14.5% and, when allowing for amino acid replacements, a 54% similarity (Fig. 6). This low level of sequence identity and homology is at the level seen between two random peptides. In contrast, most of the remaining subunits of the ATP synthase demonstrate a high degree of identity (31). For instance, the $\alpha$- and $\beta$-subunits of the yeast ATP synthase are highly conserved with percent identities of about 72 and 75%, respectively. Thus, it is surprising that $bF_6$ is able to replace subunit $h$ and form a functional enzyme, even now, knowing that they are functional homologs.

The biochemical studies here demonstrate that the complementation by $bF_6$ is due to the direct replacement of subunit $h$ with $bF_6$ and not due to a secondary mechanism. The biochemical studies indicate that $bF_6$ occupies that same spatial relationship in the yeast enzyme as subunit $h$. Cross-linking products involving subunit $h$ and subunit $d$, a component of the second stalk, were obtained from positions K96C (15) and Q203C of the latter subunit,2 which are two positions located in the hydrophilic part of subunit $d$, thus suggesting that subunit $h$ also participates to form the second stalk or stator. Bovine $F_6$ is a component of $F_0$ (11, 32), and it is associated with the stalk of the bovine coupling factor 6 is more consistent with the fact that $F_6$ also participates to form the second stalk or stator. Bovine $F_6$ is a component of $F_0$ (11, 32), and it is associated with the stalk of the bovine ATP synthase have been demonstrated to complement the corresponding null mutant strains in yeast. Expression of bovine $F_6$ and $bF_6$ are so divergent that even a one-on-one comparison of their primary structure provided no clue that they were indeed homologs. The results of this study are even more startling because these peptides are not the sole peptide in an enzyme complex, but must interact within a heterosubunit multimeric enzyme complex. The implications of this are significant because they indicate that primary structural analysis cannot be used as the sole evidence that functional peptide homologs do not exist between two species. This is true even when the peptide is within a multimeric peptide complex that otherwise might be highly conserved.

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