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SITE DIRECTED MUTAGENESIS OF THE ß-SUBUNIT OF THE YEAST MITOCHONDRIAL ATPase

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Site directed mutagenesis has been performed on the gene coding for the ß-subunit of the yeast mitochondrial F_1-ATPase. Two different regions were studied. First, the corresponding yeast amino acid, Tyr-344, which was affinity labeled in the bovine enzyme was changed to Phe-344 and Ala-344. The Phe-344 enzyme was completely active and less sensitive to the affinity reagent, 4-chloro-7-nitrobenzofurazan. In contrast, the *in vivo* level of the Ala-344 enzyme was greatly diminshed and apparently inactive. The second region studied is in the glycine rich region homologous in nucleotide binding proteins. Five different replacements were made and all mutations but one completely eliminated the biological activity and reduced the *in vivo* level of the mutant peptides. These results support the importance of these amino acids in the function of the ATPase.

The mitochondrial ATPase is composed of 5 subunits with the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (for review see 1). The ß-subunit is thought to constitute part of the active site of the enzyme, though by itself, it is unable to catalyze the rapid hydrolysis of ATP (1). Reaction of the bovine heart enzyme with the adenosine analogue, 4-chloro-7-nitrobenzofurazan (NBF-Cl) (2) and with 8-azido-ATP (3), have labeled Tyr-311 suggesting that these residues lie in or near the active site of the enzyme. To determine if this amino acid is essential, the corresponding residue it the yeast enzyme, Try-344, was altered to Ala-344 and Tyr-344 and transformed into a yeast strain that is devoid of the wild type copy. The results indicate that the phenolic hydroxyl group is not essential for activity though the residue can not be replaced with alanine.

The second region studied is a glycine rich region which is conserved in other nucleotide binding proteins including adenylate kinase, Ef-Tu and the *ras* proteins (4). This region forms the consensus sequence G X X X X G K X. In *ras* p21, a mutation of the first Gly-residue to Val makes the cell oncogenic (5) and reduces the GTPase activity of the protein (6). The role of this region is not known for all proteins though it has been shown in adenylate kinase (7), Ef-Tu (8), and the *ras* (9) proteins to form part of the nucleotide binding site. To determine if this region is

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important in the ß-subunit of the yeast mitochondrial ATPase, site directed mutagenesis has been performed on 4 of the amino acids in this region. All but one mutation inactivated the enzyme. These results indicate that this region is critical for the function of the yeast mitochondrial ATPase.

MATERIALS AND METHODS

Yeast *Saccharmoyces cerevisiae* strains DMY111-5c (MAT α leu2-3,112, trp1-289, ura3-52, atp2::LEU2), Sc293-11D1 (MAT α-ade$^-$, leu2-3,112, ura3-52, (gal1-10-7)Δ, atp2::LEU2), and Sc294 (MATα, leu2-3,112, trp1-289, ura3-52, (gal1-10-7)Δ) were used throughout this study. DMY111-5c and Sc294-11D1 are derivatives of the ATP2 null mutant, DMY111 previously described (10). Yeast mitochondria and the chloroform extracted F_1 ATPase, were prepared as described (10) from yeast grown in minimal media containing 2% galactose, 0.5% casamino acids and all of the auxotrophic requirements. ATPase activity was determined using the coupled enzyme assay at 30°C (10). Protein was determined by the method of Bradford for the water soluble protein (11) and Lowry (12) for the membrane preparations with bovine serum albumin as the standard.

Site directed mutagenesis of the gene coding for the ß-subunit of the ATPase was performed by the method of Kunkel (13) using synthetic oligonucleotides (20-25mers) prepared as described (10). After mutagenesis, the complete gene was sequenced by the method of Sanger (14) to ensure that no other mutation was present in the gene. The mutant genes were cloned into the single copy vector, Ycp50, and transformed (15) into the yeast strain, DMY111-5c. The expression of the gene was placed under the control of the GAL1 promoter by deletion mutagenesis of the ATP2 promoter with Bal31. The deleted gene was at the BamHI site of the GAL1 gene in the plasmid, pBM272 (16). These derivatives were transformed into the yeast strains Sc293-11D1 and Sc294 . In the case of Sc293-11D1, the strain transformed with the wild type ATP2 gene with the GAL1 promoter was unable to grow on medium containing galactose or glycerol as the sole carbon source. In the presence of galactose and glycerol, the strain was able to grow demonstrating the regulation of the gene expression by the presence of galactose. To test the dominance of the mutant gene products, the galactose regulated genes were transformed into the strain Sc294 which contains the wild type copy of the ATP2 gene. The yeast were grown on minimal medium containing 3% glycerol, and 3% glycerol and 1% galactose, and all the auxotrophic requirements.

RESULTS

The amino acid sequence of the regions under investigation in this study is shown in Figure 1. Figure 1A shows the homologous bovine sequence and the amino acid labeled with the affinity labels 8-azido ATP and NBf-Cl. Tyr-344 is conserved in many species (17) providing additional support to the importance of the residue. To determine the role of Tyr-344 in the active site of the protein Tyr-344 was replaced with an Ala and a Phe. Figure 1B shows the second region that was studied: the glycine rich homologous region. This region was reported by Fry et al. (4) to be conserved among many nucleotide binding proteins, including adenylate kinase and the *ras* proteins. This region is highly conserved between the ß-subunit of the ATPase of bovine and yeast. To identify the role of this region in the yeast ATPase the following independent site directed mutations were made: Val-191, Val-193, Ala-196, Arg-196, and Ser-197.

The site directed mutations were placed in a single copy vector, YCp50 and transformed into a yeast strain that was devoid of the wild type copy of the gene coding for the ß-subunit. Figure 2 shows the growth of the mutant strains on glycerol medium and on glucose medium. The only mutants that were able to grow on glycerol medium, and thus able to make ATP via oxidative-phosphorylation, were Phe-344 and Ser-197. This indicates that Tyr-344 is important for correct function of the ATP synthase and the glycine rich region is critical. However, the phenolic

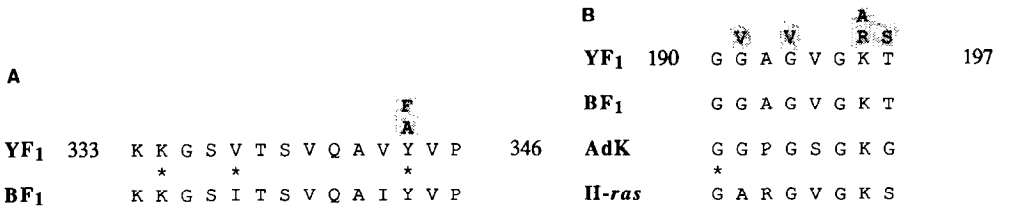

Figure 1. Site directed mutations reported in this study. Figure 1A shows the pertinent region of the sequence of the yeast and bovine ß-subunit of the mitochondrial ATPase (YF_1 and BF_1, respectively). The asterisks indicate the amino acids labeled with the affinity reagent, 8-azido-ATP (3) and NBF-Cl (2). Figure 1B shows the glycine rich region of the ß-subunit of the yeast F_1 ATPase that is homologous to other nucleotide binding proteins, including adenylate kinase (AdK) and the *ras* p21 protein (4). The asterisk indicates the glycine which when mutated to a Val causes the oncogenicity (5). The shaded amino acids are the site directed mutations reported in this study.

hydroxyl group of Tyr-344 is not essential for activity suggesting that it does not participate in any hydrogen bonding or salt bridges in the catalytic site. Tyr-344 might, however, be involved in π-π interactions with the nucleotide or another residue. The glycine rich region is very critical. Even the conservative change of Lys-196 to Arg-196 eliminated the ability of the yeast to grow on glycerol.

The mutants were determined to be recessive. This was determined by placing the gene under the control of the GAL1 promoter which is a strong promoter but active only in the presence of galactose. The mutant clones were transformed into a Gal^-, $ATP2^+$ strain. All of the transformed strains were able to grow on medium containing glycerol and galactose indicating that the mutations were recessive (data not shown).

Mitochondrial preparations were obtained from each of the yeast strain. The specific ATPase activities of the mitochondria and the chloroform extracted ATPase is shown in Table 1. Only the Phe-344 and the Ser-197 mutants had ATPase activity comparable to the wild type enzyme. The *in vivo* level of the ß-subunit was determined by immune replica analysis of the

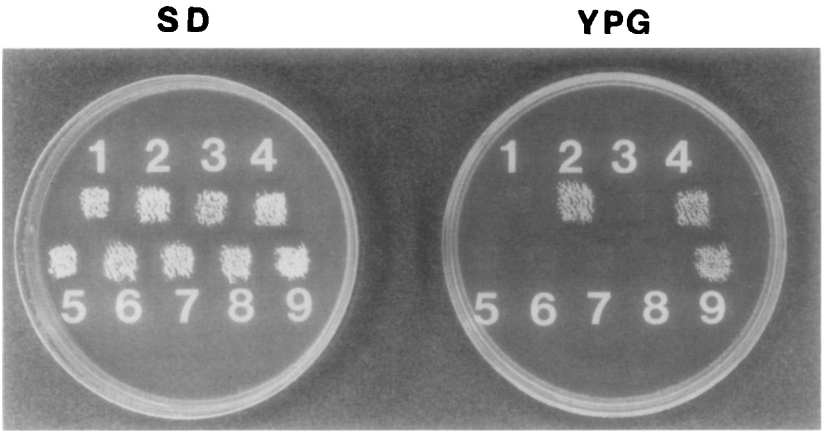

Figure 2. Growth and expression of the site directed mutations. The mutants were grown on minimal glucose medium (SD) and complete medium containing glycerol as the carbon source (YPG). The yeast strain, DMY111-5c was transformed with the plasmid YCp50 (1),with the wild type ATP2 gene (2) and with the ATP2 gene with the site directed mutations A-344 (3), F-344 (4), V-190 (5), V-193 (6), A-196 (7), R-196 (8), and S-197 (9).

Table 1. ATPase activity of the wild type and mutant enzymes. The ATPase activity was determined for the mitochondria and chloroform released enzyme ($CHCl_3$) from the yeast strain transformed with the plasmid containing the mutant ATP2 genes, as identified in the Table. The $CHCl_3$ enzyme was isolated by the minipreparation as described in the text. The activity was determined at 30°C as described in Materials and Methods.

	ATPase (μmoles/min/mg)	
Strain	Mitochondria	$CHCl_3$
Wild Type	0.62	13.0
YCp50	0.15	0.25
A-344	0.17	0.21
F-344	0.37	8.3
V-190	0.11	0.16
V-193	0.11	0.13
A-196	0.12	0.32
R-196	0.13	0.13
S-197	0.83	20.0

mitochondrial and the chloroform extracted ATPase (Figure 3). Only Phe-344 and the Ser-197 had wild type levels to the ß-subunit. All of the other strains had much reduced levels of the ß-subunit and a pleotropic effect on the level of the α-subunit.

The F_1-ATPase has been purified to homogeneity from the Phe-344 and the Ser-197 mutants. (The effect of the Ser-197 mutation on the kinetics of the enzyme has been reported elsewhere (18).) The specific activity of the Phe-344 enzyme was not significantly different from that of the wild type enzyme (140 units/min./mg protein). Nor were any of the unisite activities studied dramatically different. However, replacement of the Tyr with a Phe made the enzyme more resistant to the affinity label, NBf-Cl. Figure 4 illustrates the sensitivity of the wild type and the mutant enzymes to NBf-Cl. A number of points can be made from this Figure. First, the wild type enzyme was much more very sensitive to NBf-Cl than the mutant. Second, the addition of dithiothreitol (DTT) restored much of the activity. Third, some of the inactivation of the mutant Phe-344, is restored by the addition of DTT, but only to the level of the wild type enzyme activated with DTT. Thus, there are a number of NBf-Cl reactive residues. The major reactive residue appears to be Tyr-344 which is restored by DTT. There is a second DTT activated residue, as

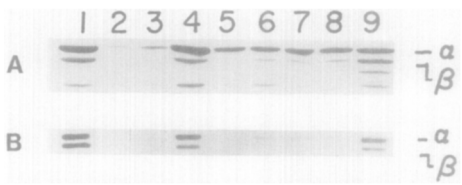

Figure 3. Level of the F_1-ATPase in the mutant strains. The strains containing each site directed mutation were grown in synthetic galactose media, the mitochondrial were isolated, and the F_1 released from the enzyme with $CHCl_3$. The total mitochondrial (A) and $CHCl_3$ proteins (B) were separated by SDS gel electrophoresis, transferred to nitrocellulose, and reacted with antibodies to the α and ß-subunits of the yeast ATPase. The yeast strain, DMY111-5C, was transformed with the plasmid YCp5O (2), with the wild type ATP2 gene (1) and with the ATP gene with the site directed mutations A-344 (3), F-334 (4), V-190 (5), V-193 (6), A-196 (7), R-196 (8), and S-197 (9). The minor bands in (A) are probably proteolytic products of the ATPase.

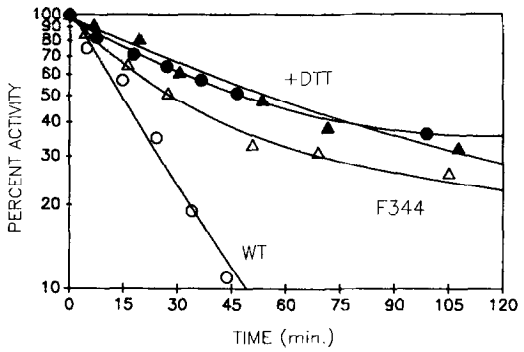

Figure 4. Sensitivity of the wild type (WT) and F344 mutant ATPase to NBf-Cl. Purified F_1 ATPase dissolved in 0.25M sucrose, 40mM 2-(N-morpholino) ethanesulfonic acid, 0.05M $MgSO_4$ (STM) at a concentration of 0.22 mg/ml. 20 nmoles (1 µl of a 20mM solution) of NBf-Cl in dimethylsulfoxide was added. At the indicated times, ATPase activity of the wild type (●,O) and mutant F-344 (▲,Δ) was determined in the absence (O,Δ) and the presence (●,▲) of 1mM dithiothreitol (DTT).

evident by the Phe-344 mutant. Finally, there are at least two other residues that are not reversibly inactivated as indicated by the nonlinear kinetics of the activity decay in the presence of DTT.

DISCUSSION

The results of this work clearly supports the importance of Tyr-344 and the glycine rich region in the function of the ß-subunit of the ATPase. The Tyr-344 is demonstrated to the major residue that is modified by the affinity reagent NBf-Cl resulting in the inactivation of the enzyme activity. This is an important conclusion since under circumstances that more than one residue is modified, a definite conclusion between reactivity and decay of enzyme activity can not be made. Further, the inactivation of Tyr-344 is reversed by the addition of DTT. The role of the phenolic hydroxyl group is not clear. Though a large amount of effort has been committed to identify a defect in the Phe-344 enzyme, no dramatic defect was observed. It is possible that the mutation caused an effect that was not yet detected, such as a change in the stability of the enzyme or a subtle change in the activity of ATP synthesis under *in vivo* conditions. Certainly, only a small selective advantage need be imparted in the wild type residue to establish its conservation between species. Similar results were also obtained with the *E. coli* enzyme (19). However, this latter work did not establish an increased resistance to NBf-Cl nor discuss the effect on the unisite ATPase parameters.

The glycine rich region is clearly important in the function of the yeast ATPase. All of the mutations except Ser-197, eliminated the *in vivo* activity and level of the ATPase. The decrease in the steady state level of the ß-subunit suggests that the ß-subunit was not folded correctly or was very unstable. This can occur by a number of mechanisms, two of which are: 1) the mutation could have caused an overall effect on the conformation of the enzyme or 2) the mutation could have eliminated the binding of ATP to the site which might be necessary for the assembly or the stability of the enzyme. The fact that none of the mutations were dominant suggests that the mutant subunits are not assembled forming hybrid molecules even under the conditions that the mutant peptide is over-expressed. However, this does not eliminate either of these possibilities.

A number of similar, but not identical mutations have been made in this region in the *E. coli* enzyme (19-22). Many of the mutations dramatically decreased the activity of the enzyme, but none of them eliminated the activity or apparently affected the steady state level of the enzyme. Some of the replacements had little effect on the activity of the enzyme and even a double mutation replacing two of the glycines with isoleucines resulted in just a 5% loss of activity. These results support the importance of this region in the activity of the enzyme. However, the yeast enzyme appears to be less tolerant of mutations in this region.

Both of the regions studied here are important in the function of the yeast ATPase. Studies being planned include the identification of the allowable amino substitutions in this region. These experiments will allow provide information on the role of the amino acids and the region in the activity of the ATPase.

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