Cyclic AMP-dependent Phosphorylation of the Precursor to β Subunit of Mitochondrial F₁-ATPase: A Physiological Mistake?

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ABSTRACT   By using the purified rat liver protein for reference in electrophoresis and peptide mapping experiments, I have identified the β subunit of mitochondrial F₁-ATPase and its cytoplasmic precursor in two-dimensional gel patterns of proteins from S49 mouse lymphoma cells. The β subunit precursor is a substrate for cAMP-dependent phosphorylation during its synthesis. Normally, both nonphosphorylated and phosphorylated forms of β subunit precursor are processed rapidly to the smaller, more acidic forms of mature β subunit. When processing is inhibited with valinomycin, both nonphosphorylated and phosphorylated forms of β subunit precursor are stabilized. Nonphosphorylated β subunit is one of the most stable of cellular proteins, but the phosphorylated form is eliminated within minutes of processing. This suggests that phosphorylated β subunit is recognized as aberrant and excluded from assembly into the ATPase complex. These results argue that cAMP-dependent phosphorylation of the β subunit precursor is a physiological mistake that is remedied after mitochondrial import and processing.

Over the past several years specific protein phosphorylation has been implicated increasingly in cellular regulation by effectors varying from hormones and growth factors to transforming gene products of tumor viruses (reviewed in reference 1). In trying to assess mechanisms by which protein kinases regulate cell function, it has become important to decide whether or not physiological regulation of a particular substrate phosphorylation must be taken as *prima facie* evidence for the physiological significance of the phosphorylation. This report presents data supporting an accidental basis for at least one cAMP-regulated phosphorylation in intact cells. The strong possibility of such an error suggests that caution should be exercised in assigning physiological significance to phosphorylations for which functional roles have not been demonstrated.

Previous studies using two-dimensional gel electrophoresis to separate phosphorylated from nonphosphorylated forms of substrate proteins revealed a class of cAMP-dependent phosphorylations that are restricted to protein nascent chains (2–4). These phosphorylations are generally substoichiometric, and they tend to be more stable than the more orthodox posttranslational phosphorylations. In principle, such nascent chain phosphorylations could provide cells with a record of prior kinase activation, but they could as easily represent mistakes of no functional importance. In this report, I identify one substrate for cAMP-dependent nascent chain phosphorylation as the cytoplasmic precursor to β subunit of mitochondrial F₁-ATPase and show that the resulting phosphorylated form of mature β subunit is rapidly eliminated from cells. The small proportion of β subunit affected by cAMP-dependent phosphorylation and the rapid elimination of phosphorylated β subunit argue strongly for the accidental nature of the modification.

β subunit belongs to a class of cytoplasmically-synthesized mitochondrial proteins that are imported into mitochondria by an energy-dependent process that includes processing by a specific protease of the mitochondrial matrix (5–7). Because import requires a mitochondrial membrane potential (8), radiolabeling of the mature forms of such proteins can be inhibited by a variety of antimitochondrial agents (including nonactin, valinomycin, dinitrophenol, oligomycin, antimycin, and rhodamine [9]). An acidic protein of M_r ~52,000, whose labeling in human lymphoblastoid cells is inhibited by such agents, was tentatively identified as β subunit of F₁-ATPase (9) on the basis of its high abundance and molecular weight. Here I confirm the identification of β subunit with this valinomycin-sensitive protein in cultured S49 mouse lymphoma cells. Furthermore, β subunit and its larger pre-

cursor are shown to correspond to species identified previously as substrates for cAMP-dependent nascent chain phosphorylation (2, 3).

MATERIALS AND METHODS

Chemicals, Radiochemicals, and Film: Emetine, D,L-isoproterenol, and papain (type III, 2× crystallized) were obtained from Sigma Chemical Co. (St. Louis, MO), protease from *Staphylococcus aureus*, strain V8 was obtained from Miles Laboratories (Elkhart, IN), and valinomycin was obtained from Calbiochem-Behring Corp. (San Diego, CA). Purified type II F_1-ATPase from rat liver mitochondria (10) was a gift from Dr. Robert J. Fisher (Boston Biomedical Institute). [^{35}S]Methionine and ACS scintillation mixture were purchased from Amersham Corp. (Arlington Heights, IL); x-ray film (type XAR) and photographic chemicals were bought from Eastman Kodak (Rochester, NY). Chemicals used for gel electrophoresis were obtained as reported previously (11); all other chemicals were reagent grade and used without further purification.

Cells: Wild-type S49 cells (subline 24.3.2) were grown in suspension culture in Dulbecco's modified Eagle's Medium with 10% heat-inactivated horse serum as described previously (12).

Radiolabeling and Preparation of Cell Extracts: Cells were labeled with [^{35}S]methionine in low methionine media as described previously (2), except that, for the experiments of Figs. 3–5, methionine concentration was reduced from 5 to 2.5 μM, HEPES concentration was increased from 10 mM to 25 mM, and cell density was increased from 2.5 × 10^6 to 2.5 × 10^7. For the experiment of Fig. 1, cells were labeled for 30 min with [^{35}S]methionine at 250 μCi/ml after 2 h preincubation in low methionine medium with or without 10 μM valinomycin. For the experiment of Fig. 3, cells were incubated 16 min in low methionine medium with or without 10 μM valinomycin, then labeled for 2 min with [^{35}S]methionine in the presence or absence of 10 μM isoproterenol; [^{35}S]methionine was at 0.5 mCi/ml for samples without valinomycin and at 1 mCi/ml for samples with the inhibitor. For the experiment of Fig. 4, cells were treated for 1 min with 10 μM isoproterenol after 13 min preincubation, then labeled for 5 min with [^{35}S]methionine at 0.5 mCi/ml. For the label-chase experiment of Fig. 5, cells were preincubated in low methionine medium for 25 min; isoproterenol was added to 10 μM; and, after 1 min, [^{35}S]methionine was added to 0.5 mCi/ml. After 2 min of labeling, cells were diluted with 9 vol of conditioned growth medium (2) containing 125 μM emetine, and portions were incubated with or without 10 μM valinomycin. Samples were harvested by centrifugation for 5 s at 10,000 g in a Fisher microcentrifuge (Fisher Scientific Corp., Pittsburgh, PA) (after dilution with ice-cold phosphate-buffered saline containing 2 mM L-methionine for the more concentrated cell suspensions of the experiments of Figs. 3 and 4); supernatant fractions were aspirated; and cell pellets were dissolved in gel sample buffer containing extra urea (2) using 25 μl/6.25 × 10^5 cells to give extracts containing ~0.75 mg/ml protein. Extracts were frozen and stored at −70°C.

PAGE: The O'Farrell two-dimensional gel procedure (13) was used with modifications described previously (2). Second dimension SDS gels were 7.5% in polyacrylamide. For the peptide maps of Fig. 2, a and d, unlabeled S49 cells were extracted in gel sample buffer and portions of the extract were subjected to two-dimensional electrophoresis. Mitcon:1 was excised from a gel of ~100 μg of S49 cell protein, and mitcon:2 was excised from a gel of ~50 μg of protein. For Fig. 2, b and c, ~0.6 μg portions of pure rat liver F_1-ATPase were subjected to one-dimensional SDS gel electrophoresis (14) on a 7.5% polyacrylamide gel to separate α and β subunit chains. Gel pieces containing about half the α or β subunit bands were excised for peptide mapping. Gels from which species were to be excised were stained very briefly (~2 min) with 0.2% Coomassie Blue in 50% trichloroacetic acid, destained for 1 h using two changes of 7% acetic acid, and then dried. Two-dimensional gel patterns of [^{35}S]methionine-labeled proteins were revealed by direct autoradiography.

Peptide Mapping: Species excised from gels were subjected to partial proteolysis peptide mapping following the method of Cleveland et al. (15) with modifications to be described in detail elsewhere.[1] Rehydrated pieces from dried gels were aligned on the stacking layer of a 15% SDS polyacrylamide gel, fixed in place with a small volume of melted agarose in a modified SDS gel sample buffer, and overlaid with protease in the melted agarose solution. About 0.05 ml of protease solution were used per centimeter of slab gel. Digestions were allowed to proceed in the gel stacking layer (15). Protease concentrations are noted in figure legends. The peptide mapping gel of Fig. 2 was stained with silver using the procedure of Merril et al. (16). The peptide patterns of the gels

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[1] Steinberg, R. A. 1984. Mapping endpoints of partial proteolysis fragments from regulatory subunit of type I cyclic AMP-dependent protein kinase *Anal. Biochem.* In press.
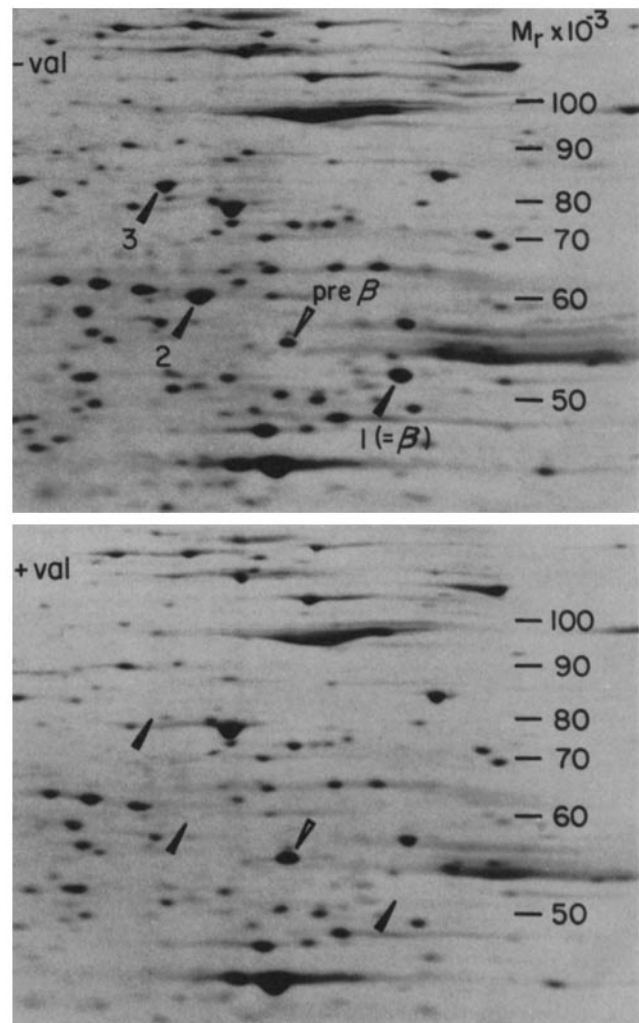

FIGURE 1 Valinomycin-mediated inhibition of mitochondrial protein labeling. S49 cells were preincubated for 2 h with or without valinomycin (*val*) and then labeled for 30 min with [^{35}S]methionine. 10^6 counts per minute of acid-precipitable radioactivity were subjected to two-dimensional gel electrophoresis. Patterns shown are from two day autoradiographic exposures. Filled arrowheads indicate positions of the valinomycin-sensitive proteins, mitcon:1 (= β subunit of F_1-ATPase; see text), mitcon:2, and mitcon:3; open arrowheads indicate the position of the putative precursor to β subunit of F_1-ATPase.

of Fig. 4 were revealed by fluorography following the procedure of Bonner and Laskey (17).

RESULTS

Fig. 1 shows two-dimensional gel patterns of S49 cell proteins labeled with [^{35}S]methionine in the absence or presence of valinomycin. As described previously for human lymphoblastoid cells (9), labeling of three prominent proteins (and a number of more minor species) was totally inhibited by the antimitochondrial agent. These species, labeled 1 (equivalent to β), 2, and 3 correspond in both relative labeling and electrophoretic positions to the major mitochondrial proteins identified as mitcon:1, mitcon:2, and mitcon:3 by Anderson in human cells (9). Mitcon:1 and mitcon:2 had been tentatively designated as the α and β subunits of F_1-ATPase on the bases of their high abundances and apparent molecular weights. Co-electrophoresis of labeled S49 cell proteins with F_1-ATPase purified from rat liver mitochondria revealed that

α subunit was significantly smaller and more basic than mitcon:2, while β subunit was slightly smaller than mitcon:1 and about one charge unit more acidic (data not shown). Since our previous studies had shown that mitcon:1 (non-phosphorylated protein K—see below) from mouse cells was more basic by about a single charge unit than the corresponding species from rat cells (4, 18), the electrophoretic behavior of purified rat β subunit supported its identification with mitcon:1. This assignment was strengthened by studies using antibodies against rat F_1-ATPase to precipitate labeled S49 cell proteins, but partial proteolysis of the S49 cell proteins compromised the results (not shown). Confirmation was provided by partial proteolysis mapping of gel-purified rat β subunit and S49 mitcon:1. Fig. 2 shows gel patterns of peptides generated by papain from α or β subunit of rat F_1-ATPase, and from S49 mitcon:1 and mitcon:2. There was no apparent relationship between mitcon:2 and α subunit or between α and β subunits, but the peptide patterns from β subunit and mitcon:1 were strikingly similar. Slight differences in mobilities of some peptides from the two proteins were consistent with proteolytic clipping near one end of the rat protein during purification. Peptide patterns generated by protease from *S. aureus*, strain V8 also supported the identification of mitcon:1 with β subunit (not shown).
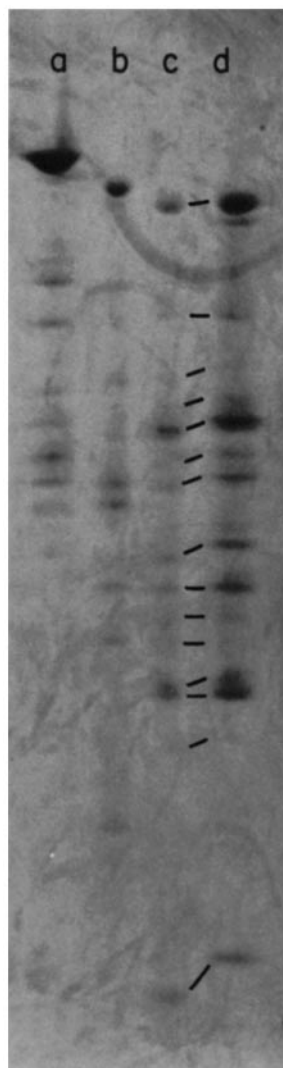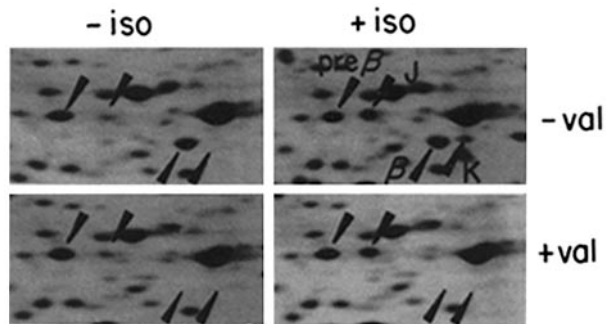

FIGURE 3   Cyclic AMP-dependence and valinomycin-sensitivity of putative phosphorylated forms of β subunit and its precursor. S49 cells were labeled for 2 min with [^{35}S]methionine in the absence or presence of isoproterenol (*iso*) and/or valinomycin (*val*). 4×10^5 acid-precipitable counts per minute from cell extracts were subjected to two-dimensional gel electrophoresis. Arrowheads indicate positions of β subunit, its presumed precursor (pre-β), and the cAMP-dependent forms, proteins J and K, in autoradiographic patterns resulting from 14-d exposures.

FIGURE 2   Partial proteolysis peptide maps of mitcon:1 and mitcon:2 from S49 cells and of α and β subunits from purified rat liver mitochondrial F_1-ATPase. Protein species purified by one- or two-dimensional gel electrophoresis were digested with papain in an SDS polyacrylamide slab gel as described in Materials and Methods. The overlay solution contained papain at a concentration of 50 ng/ml. Peptide patterns were revealed by silver staining. (a) S49 mitcon:2 (b) α subunit of rat liver mitochondrial F_1-ATPase; (c) β subunit of mitochondrial F_1-ATPase; (d) S49 mitcon:1.
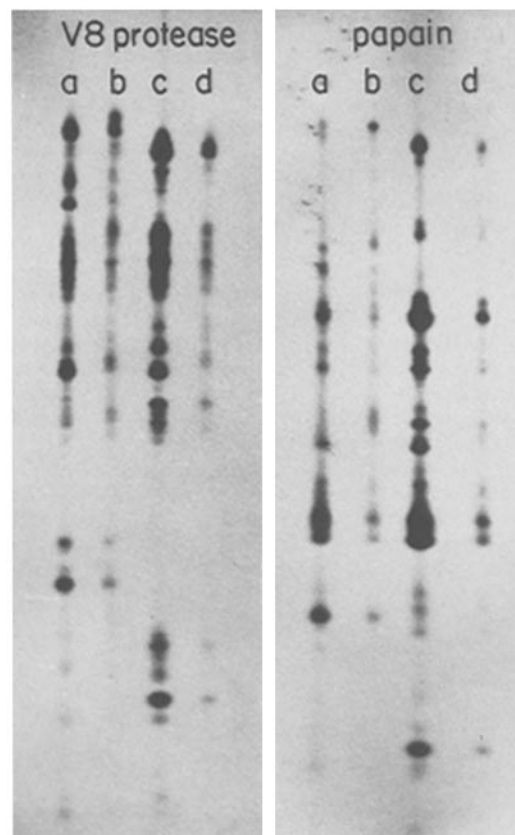

FIGURE 4   Peptide maps of cAMP-dependent and -independent forms of β subunit and its putative precursor. S49 cells were labeled for 5 min with [^{35}S]methionine in the presence of isoproterenol, and labeled proteins were separated by two-dimensional gel electrophoresis using 2×10^6 counts per minute of acid-precipitable material per gel. Species indicated by arrowheads in Fig. 3 were excised from dried gels and subjected to partial proteolysis mapping as for Fig. 2 but using either 50 µg/ml protease from *S. aureus*, strain V8 (V8 protease) or 10 ng/ml papain in the agarose overlay. Fluorographic exposures were for 10 d. (a) pre-β; (b) protein J; (c) β subunit; (d) protein K.

My interest in the identity of mitcon:1 derived from studies showing that a more acidic satellite spot appeared in gel patterns from cells labeled with [^{35}S]methionine in the presence of a variety of agents that activated intracellular cAMP-dependent protein kinase. Appearance of this species (called protein K in earlier studies by P. Coffino and myself [2, 3]) required that the cells had a functional cAMP-dependent protein kinase and that kinase activation was concurrent with protein labeling. We concluded, therefore, that the species represented a phosphorylated form of a protein (either β subunit or a species comigrating with β subunit) that was susceptible to cAMP-dependent modification only during its synthesis. Another cAMP-dependent species (called protein J) had electrophoretic properties similar to those described by Anderson for the cytoplasmic precursor to mitcon:1 (designated pre-β in Fig. 1 [9]). The nonphosphorylated and phosphorylated forms of protein J or pre-β and the phosphorylated form of protein K were metabolically labile, while mature β subunit was stable (2; and below).

Fig. 3 shows the cAMP-dependent appearance of the putative phosphoproteins J and K. In the presence of isoproterenol, a potent inducer of intracellular cAMP, significant labeling of J and K was observed, but, in the absence of isoproterenol, negligible labeling of J and K was seen. When cells were labeled in the presence of valinomycin, neither β subunit nor protein K were detected. The sensitivities of protein K and mature β subunit to inhibition by valinomycin suggested that both proteins were generated by mitochondrial processing of cytoplasmic precursors and supported, therefore, the notion that K was a phosphorylated form of β

subunit. Fig. 4 shows gel patterns of peptides generated from the presumed phosphorylated and nonphosphorylated forms of pre-β (J) and β subunit (K) by digestion with either protease from *S. aureus*, strain V8 (V8 protease) or papain. These patterns confirmed the close relationship of pre-β and mature β subunit and showed that the cAMP-dependent forms (J and K) were homologous to their cAMP-independent counterparts (pre-β and β). These results suggested the following scheme for the appearance of the various forms of pre-β and β subunit: a fraction of pre-β molecules were phosphorylated during translation in the presence of active cAMP-dependent protein kinase; both phosphorylated and nonphosphorylated forms of pre-β were imported into mitochondria; and both forms were cleaved to mature forms by the mitochondrial protease specific for this process (7).

The precursor/product relationship of pre-β and β was corroborated by the experiment of Fig. 5, which also assessed the fate of phosphorylated β subunit. Cells labeled briefly in the presence of isoproterenol were mixed with emetine to inhibit further protein synthesis, and then were incubated in the presence or absence of valinomycin. In the absence of valinomycin the nonphosphorylated form of pre-β disappeared with a half-time on the order of 1 to 2 min, and the phosphorylated form disappeared, albeit a bit more slowly. Consistent with their being precursors to the two forms of β subunit, both forms of pre-β were markedly stabilized by valinomycin. With or without valinomycin the phosphorylated form of mature β subunit disappeared with a half-time of about 1 to 2 min, while the nonphosphorylated form was completely stable. In separate experiments (not shown) la-

FIGURE 5 Stabilities of cAMP-dependent and -independent forms of β subunit and its precursor in the presence and absence of valinomycin. Cells were labeled for 2 min with [^{35}S]methionine in the presence of isoproterenol, diluted into medium containing emetine, and then divided into two portions, of which one was treated with valinomycin (val). At the times indicated, samples were harvested for analysis by two-dimensional gel electrophoresis. 4 × 10^5 counts per minute of cellular proteins were loaded onto each gel, and autoradiographs were exposed for 14 d. Arrowheads indicate the positions of pre-β, protein J, β subunit, and protein K as in Fig. 3.
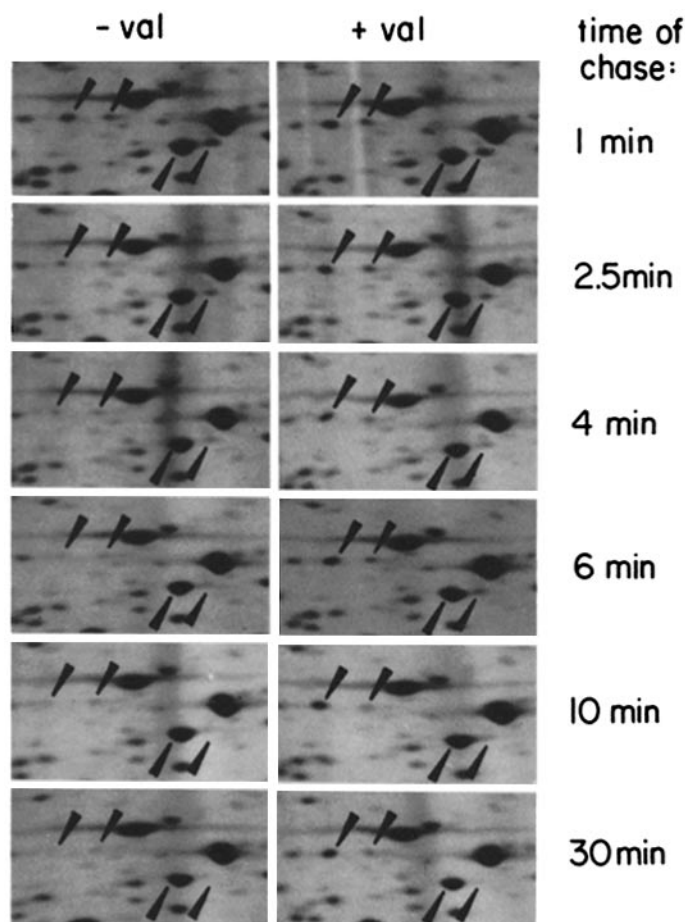
beled β subunit did not decay perceptibly over chase periods as long as 21 h.

DISCUSSION

The results presented above and previously (2-4) show that nascent chains of the cytoplasmic precursor to β subunit of mitochondrial F_1-ATPase are substrates for cAMP-dependent phosphorylation and that this modification has little if any effect on mitochondrial import and processing of β subunit. The instability of phosphorylated mature β subunit suggests that it is recognized as aberrant and eliminated, perhaps by the ATP-dependent mitochondrial protease that has been implicated in rapid degradation of incomplete mitochondrial proteins generated by treatment with puromycin (19, 20). Exclusion of aberrant subunits of a mitochondrial protein at the time of oligomer assembly has also been proposed recently to explain the absence of mutant forms of ornithine transcarbamylase in liver mitochondria from mice heterozygous for a mutation causing abnormal splicing of messenger RNA for the enzyme (21). Alternatively, elimination of modified β subunit could be by dephosphorylation. The small proportion of pre-β phosphorylated under the most favorable conditions made it impossible to judge between these alternatives. Since disappearance of phosphorylated β subunit was unaffected by valinomycin (Fig. 5), it appears to be independent of the mitochondrial membrane potential.

The enormous difference in stabilities between nonphosphorylated and phosphorylated forms of β subunit ensures that there is no accumulation of phosphorylated subunit in mitochondria. Therefore, a functional role for β subunit phosphorylation based on different activities of phosphorylated and nonphosphorylated forms of the protein is unlikely. Furthermore, the very limited extent of pre-β phosphorylation and the apparent absence of posttranslational phosphorylation of either pre-β or mature β subunit make it improbable that phosphorylation functions to regulate levels of mitochondrial β subunit. Taken together, these propositions argue against the physiological importance of cAMP-regulated β subunit modification. Reports of enzyme phosphorylations that do not affect enzyme activities (reviewed in reference 22) suggest that accidental protein phosphorylation may be a common physiological occurrence. So long as such errors do not interefere with protein function, they are likely to be tolerated.

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