Properties of Bound Inorganic Phosphate on Bovine Mitochondrial F_{1}F_{0}-ATP Synthase

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Beef-heart mitochondrial F_{1}F_{0}-ATP synthase contained six molecules of bound inorganic phosphate (P_{i}). This phosphate exchanged completely with exogenous ^{32}P_{i} when the enzyme was exposed to 30% (v/v) dimethyl sulfoxide (DMSO) and then returned to a DMSO-free buffer (Beharry and Bragg 2001). Only two molecules were replaced by ^{32}P_{i} when the enzyme was not pretreated with DMSO. These two molecules of ^{32}P_{i} were not displaced from the enzyme by the treatment with 1 mM ATP. Similarly, two molecules of bound ^{32}P_{i} remained on the DMSO-pretreated enzyme following addition of ATP, that is, four molecules of ^{32}P_{i} were displaced by ATP. The ATP-resistant ^{32}P_{i} was removed from the enzyme by pyrophosphate. It is proposed that these molecules of ^{32}P_{i} are bound at an unfilled adenine nucleotide-binding noncatalytic site on the enzyme. Brief exposure of the enzyme loaded with two molecules of ^{32}P_{i} to DMSO, followed by removal of the DMSO, resulted in the loss of the bound ^{32}P_{i} and in the formation of two molecules of bound ATP from exogenous ADP. A third catalytic site on the enzyme was occupied by ATP, which could undergo a P_{i} \rightarrow ATP exchange reaction with bound P_{i}. The presence of two catalytic sites containing bound P_{i} is consistent with the X-ray crystallographic structure of F_{1} (Bianchet, et al., 1998). Thus, five of the six molecules of bound P_{i} were accounted for. Three molecules of bound P_{i} were at catalytic sites and participated in ATP synthesis or P_{i} \rightarrow ATP exchange. Two other molecules of bound P_{i} were present at a noncatalytic adenine nucleotide-binding site. The location and role of the remaining molecule of bound P_{i} remains to be established. We were unable to demonstrate, using chemical modification of sulfhydryl groups by iodoacetic acid, any gross difference in the conformation of F_{1}F_{0} in DMSO-containing compared with DMSO-free buffers.

KEY WORDS: ATP synthase; adenine nucleotides; bound phosphate; phosphate exchange; ATP synthesis; mitochondria; F_{1}F_{0} catalytic sites.

INTRODUCTION

The proton-translocating ATP synthase is the terminal enzyme of oxidative phosphorylation in which process it synthesizes ATP from ADP and inorganic phosphate. ATP synthase can be separated into two parts. The F_{1} portion has the catalytic machinery for the synthesis and hydrolysis of ATP (Boyer, 1997; Hatefi, 1993; Pedersen and Amzel, 1993; Penevsky and Cross, 1991). The F_{0} portion is an intrinsic membrane protein composed of nine types of subunits in mammalian mitochondrial F_{1}F_{0}-ATP synthase (Collinson et al., 1994a). It contains the pathway of proton translocation. The three-dimensional structures of bovine and rat liver mitochondrial F_{1} and of yeast F_{0} have been partly determined (Abrahams, et al., 1994; Bianchet et al., 1998; Stock et al., 1999). F_{1} is readily purified in a soluble form with high ATPase activity. It has six binding sites for adenine nucleotide, of which three sites have a direct role in catalysis. The remaining three sites (noncatalytic sites) may be structural or may have an indirect role in ATP hydrolysis/synthesis. Purified F_{1}, as isolated, generally contains 3 mol bound adenine nucleotide/mol enzyme. Kironde and
Cross (1986) proposed that two molecules of nucleotide are bound at noncatalytic sites and one molecule at a catalytic site. Five to six moles of bound P_i/mole of F_1 are present on bovine mitochondrial and E. coli F_1 (Beharry and Bragg, 1991a, 1992a). Penefsky (1977) and Kasahara and Penefsky (1978) found a single high-affinity (K_D, 80 μM) binding site for [32P]phosphate which they suggested is a catalytic site. A second nonsaturable binding site was detected. The [32P]phosphate, which was bound in Penefsky and Kasahara’s experiment, presumably exchanged with endogenous bound phosphate.

Purified soluble F_1 F_0 has lower ATPase activity than F_1. We have shown that a purified soluble preparation of bovine mitochondrial F_1 F_0 contained 2 mol ATP and 2 mol ADP/mol enzyme (Beharry and Bragg, 1996). Three of the four bound adenine nucleotides were exchangeable on incubation with MgATP. Bound ATP (1 mol/mol F_1 F_0) was readily lost from the enzyme on passage of the F_1 F_0 through a centrifuged column of Sephadex G-50. It was initially concluded (Beharry and Bragg, 1996) that F_1 F_0 differed from F_1 in having all of its noncatalytic sites occupied by adenine nucleotide (2 mol ATP and 1 mol ADP). The ADP, which was readily lost from the enzyme, presumably occupied a catalytic site. However, our recent work (Beharry and Bragg, 2001) has shown that one of the molecules of bound ATP is present at a catalytic site in F_1 F_0. Thus, F_1 F_0 contains 1 molecule ATP and 1 molecule ADP at both the catalytic and the noncatalytic sites.

F_1 F_0, as prepared, also contains six molecules of bound Pi (Beharry and Bragg, 2001). Incubation of F_1 F_0 with [32P]Pi in 30% (v/v) DMSO resulted in the exchange of all six molecules of nonradioactive Pi by [32P]Pi, Adenine nucleotides were also lost with the sole retention of a single molecule of ATP at a catalytic site (Beharry and Bragg, 2001). Two of the six molecules of phosphate present on the DMSO-treated enzyme were reactive following transfer of the F_1 F_0 to a DMSO-free buffer. One molecule reacted with bound ATP in a [32P]Pi ↔ ATP exchange reaction. A further molecule phosphorylated exogenous ADP to give bound [32P]ATP (Beharry and Bragg, 2001). Thus, like F_1, F_1 F_0 can be induced to form ATP by exposure to organic solvents like DMSO (Beharry and Bragg, 1991a,b, 1992a–d; Sakamoto, 1984a,b; Sakamoto and Tonomura, 1983; Cross et al., 1984; Gomez-Puyou et al., 1986; Kandpal et al., 1987; Tuesta de Gomez-Puyou et al., 1993, 1995, 1998, 1999; Yoshida, 1983; Yoshida and Allison, 1986). There was a significant difference between F_1 and F_1 F_0 in the effect of DMSO in inducing formation of ATP at catalytic sites. DMSO had to be present during the reaction for F_1 to make ATP. By contrast, DMSO had to be removed from the system before F_1 F_0 could form ATP (Beharry and Bragg, 2001).

In contrast to the behavior of the DMSO-pretreated enzyme, incubation of F_1 F_0 with [32P]Pi in a DMSO-free buffer replaced only two molecules of bound Pi by [32P]Pi (Beharry and Bragg, 2001), in agreement with the results of Penefsky (1977) and Kasahara and Penefsky (1978) with F_1. The F_1 F_0 synthase retained 1 molecule each of ATP and ADP at noncatalytic sites, and one molecule of ATP at a catalytic site. The two molecules of [32P]Pi did not undergo the [32P]Pi ↔ ATP exchange reaction or form ATP with exogenous ADP (Beharry and Bragg, 2001).

In this paper, we further characterize the properties of the bound Pi of the F_1 F_0-ATP synthase.

MATERIALS AND METHODS

Materials

The sources of chemicals and enzymes have been given (Beharry and Bragg, 1996). Additional materials were obtained from the companies indicated: Sigma (glucose-6-phosphate dehydrogenase; HEPES; polyethyleneimine cellulose plates); Amersham ([P32]orthophosphate); Brinkman (polyethyleneimine cellulose plates); Pierce Chemical Company (BCA protein assay kit).

Preparations and Assays

The F_1 F_0-ATP synthase was solubilized from bovine submitochondrial particles with deoxycholate and purified by chromatography on a column of DEAE-Sephadex CL-6B in the presence of reduced Triton X-100 and MgCl_2, as described (Beharry and Bragg, 1996). Azide was omitted. The specific activity was in the range of 3 to 7 μmol ATP hydrolyzed/min/mg protein. The enzyme was at least 90% pure. SDS/PAGE gels showed α, β, γ, δ, ε, α, β, c, d, e, OSCP, F_0, and A6L subunits were present, as also was the inhibitor protein. The ATPase activity of the synthase was inhibited 80% by 125 μg/ml oligomycin.

Enzyme assays, protein determination by the Lowry method, gel electrophoresis, nucleotide binding and chase studies, luminometric measurements of bound ADP and ATP, Sephadex G-50 centrifuged columns, were carried out as previously described (Beharry and Bragg, 1996). Protein was also determined by the bichinchonic acid (BCA) method, as described by the manufacturer (Pierce Chemical Co.), since this did not give a high background reading with DMSO. Both methods were equally suitable.