# Regulation of Cytochrome *c* Oxidase by Interaction of ATP at Two Binding Sites, One on Subunit VIa†

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ABSTRACT: Cytochrome *c* oxidase isolated from a wild-type yeast strain and a mutant in which the gene for subunit VIa had been disrupted were used to study the interaction of adenine nucleotides with the enzyme complex. At low ionic strength (25 mM potassium phosphate), in the absence of nucleotides, the cytochrome *c* oxidase activity of the mutant enzyme lacking subunit VIa was higher than that of the wild-type enzyme. Increasing concentrations of ATP, in the physiological range, enhanced the cytochrome *c* oxidase activity of the mutant much more than the activity of the wild-type strain, whereas ADP, in the same concentration range, had no significant effect on the activity of the cytochrome *c* oxidase of either strain. These results indicate an interaction of ATP with subunit VIa in the wild-type enzyme that prevents the stimulation of the activity observed in the mutant enzyme. The stimulation of the mutant enzyme implies the presence of a second ATP binding site on the enzyme. Quantitative titrations with the fluorescent adenine nucleotide analogues 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) and 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP) confirmed the presence of two binding sites for adenine nucleotides per monomer of wild-type cytochrome *c* oxidase and one binding site per monomer of mutant enzyme. Covalent photolabeling of yeast cytochrome *c* oxidase with radioactive 2-azido-ATP further confirmed the presence of an ATP binding site on subunit VIa. Labeling of both tissue specific isoforms of bovine cytochrome *c* oxidase in subunit VIa indicated that the ATP binding site is conserved in the subunit from different species as well as in different isoforms. Since the C-terminal part of subunit VIa, which is located in the intermembrane space, is much more strongly conserved than the N-terminal part of the polypeptide, the labeling results suggest a common ATP binding site located at the C-terminal part of the polypeptide. Taken together, these observations support a regulatory role for subunit VIa of cytochrome *c* oxidase in which this subunit monitors the concentration of ATP in the intermembrane space and inhibits the enzyme activity at physiological concentrations of ATP.

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal enzyme complex of the respiratory chain located in the mitochondrial inner membrane of eukaryotic cells and in the plasma membrane of bacteria. The enzyme couples the transfer of electrons from ferrocytochrome *c* to molecular oxygen with the translocation of protons across the membrane. The transmembrane proton gradient thus formed is used to drive the synthesis of ATP and ion transport (Hatefi, 1985). In eukaryotes, the three largest subunits of cytochrome *c* oxidase (I, II, and III) are encoded on the mitochondrial genome. These subunits are thought to be crucial for the catalytic functions of the enzyme because they are associated with the prosthetic groups, and homologues of these subunits compose the $aa_3$-type cytochrome *c* oxidases of bacteria (Capaldi, 1990; Cooper et al., 1991). In addition to the subunits of mitochondrial origin, the eukaryotic enzyme contains a number of subunits coded for in the nucleus. This number may vary somewhat with species, but studies of subunit structure are complicated by the fact that the composition of the isolated enzyme depends on the purification procedure used (Capaldi, 1990; Taanman & Capaldi, 1992). The function(s) of the nuclear-coded subunits is still a matter of debate. It has been proposed that these subunits are involved both in the assembly of the complex and in the regulation of enzyme activity via allosteric modification (Kadenbach, 1986; Poyton et al., 1988; Capaldi, 1990; Cooper et al., 1991). The presence of isoforms of some of the nuclear-encoded subunits is considered support for the idea that these polypeptides play a regulatory role.

We have chosen a genetic approach in the yeast *Saccharomyces cerevisiae* (Aggeler & Capaldi, 1990; Lightowlers et al., 1991; Taanman & Capaldi, 1993) to investigate the function(s) of the nuclear-encoded subunits. Yeast cytochrome *c* oxidase contains at least eight nuclear-encoded subunits and closely resembles the subunit structure of the mammalian enzyme (Taanman & Capaldi, 1992). Gene disruption (*null*) mutants have now been constructed for every one of the nuclear-encoded subunits in yeast. Studies of these mutants have revealed that disruptions in the genes for the subunit isoforms Va and Vb or for the subunits IV, VI, VII, or VIIa impair assembly of the enzyme (Poyton et al, 1988; Aggeler & Capaldi, 1990), precluding further analysis of catalytic functions. A *null* strain for subunit VIb grew poorly on nonfermentable carbon sources and had a markedly diminished cytochrome *c* oxidase activity (LaMarche et al., 1992). Since fully active cytochrome *c* oxidase can be isolated from yeast without subunit VIb (Power et al., 1984), this subunit must also have some role in the assembly of the enzyme complex. In a *null* strain for subunit VIII, cytochrome *c* oxidase activity was reduced by only 20% compared to its parental strain (Patterson & Poyton, 1986), indicating that subunit VIII has a minimal role in the assembly of, the homeostasis of, or catalysis by the enzyme.

Recently, we isolated the gene for cytochrome *c* oxidase subunit VIa in yeast, the homologue of subunit VIa in mammals (Taanman & Capaldi, 1993). This subunit occurs

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as two isoforms in mammals, which are expressed in a tissue specific manner [see, for example, Merle and Kadenbach (1980), Yanamura et al. (1988), Ewart et al. (1991), and Taanman et al., 1992, 1993]. Chemical labeling, protease digestion, and monoclonal antibody mapping studies with the bovine enzyme have demonstrated that subunit VIa spans the membrane once and has its C-terminus in the intermembrane space (Zhang & Capaldi, 1988; Zhang et al., 1988, 1991). A *null* mutant for subunit VIa in yeast showed a slightly reduced growth rate on nonfermentable carbon sources (Taanman & Capaldi, 1993). Heme spectra and analysis of immunopurified cytochrome *c* oxidase from this mutant demonstrated that the enzyme is fully assembled without subunit VIa. In cytochrome *c* oxidase activity assays with solubilized mitochondria performed spectrophotometrically, increasing concentrations of ATP were found to stimulate the activity of the *null* mutant to a much higher extent than that observed for the parental strain (Taanman & Capaldi, 1993). These results suggest that ATP interacts with subunit VIa and thereby modulates the enzyme activity.

To follow up this earlier study, we have examined the interaction of isolated yeast cytochrome *c* oxidase with ATP, ADP, the fluorescent ATP analogue 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP[1]), TNP-ADP, and the photoaffinity label 2-azido-ATP (2-$N_3$ATP). We provide clear evidence that (1) subunit VIa contains a binding site for adenine nucleotides, (2) there is a second adenine nucleotide binding site on the enzyme, and (3) these two sites are apparently working in competition with each other in modulating the electron transfer activity. Our results are compared with the variable observations that have been made on nucleotide effects with mammalian cytochrome *c* oxidase.

## MATERIALS AND METHODS

*Chemicals.* ATP and ADP disodium salts were purchased from Sigma Chemical Co. TNP-ATP and TNP-ADP disodium salts were obtained from Molecular Probes Inc., and their purity was checked by thin-layer chromatography on poly(ethylene imine) cellulose plates from Sigma Chemical Co. as described previously (Grubmeyer & Penefsky, 1981). The analysis indicated that approximately 5% of the TNP-ATP preparation was hydrolyzed to TNP-ADP. [$\beta,\gamma$-$^{32}$P]-2-$N_3$ATP, with a specific activity of 316 $\mu$Ci/$\mu$mol, was the kind gift of Dr. R. L. Cross (State University of New York at Syracuse). *n*-Dodecyl $\beta$-D-maltoside (lauryl maltoside) was purchased from Anatrace. D-(+)-Raffinose pentahydrate was obtained from Wako Chemicals USA Inc. Cytochrome *c*, isolated from *Saccharomyces cerevisiae*, was purchased from Sigma. Cytochrome *c* was fully reduced to ferrocytochrome *c* with sodium dithionite and then separated from the reductant by passage through a Sephadex G-25M column (PD10, Pharmacia LKB Biotechnology Inc.), equilibrated with 25 mM potassium phosphate (pH 6.2). Aliquots of the ferrous form were stored at −80 °C. Trypsin (type XIII) was obtained from Sigma and used as a 0.2 mg/mL stock solution in water. Poly(vinylidine difluoride) (PVDF) membrane (Immobilon-P) was purchased from Millipore. All other chemicals were of the highest purity commercially available.

*Antibodies.* Monoclonal antibodies against yeast cytochrome *c* oxidase subunit VIa (6F10) and bovine cytochrome *c* oxidase subunit VIa-H (4H2-A5) and the polyclonal antiserum against bovine cytochrome *c* oxidase subunit VIa-L have been described previously (Taanman & Capaldi, 1993; Taanman et al., 1993). Monoclonal antibody 11D11-H6, against yeast cytochrome *c* oxidase subunit VI, was raised against the isolated subunit as described (Taanman & Capaldi, 1993). Monoclonal antibodies 8F2-E3 and 3G5-F7-G3, against bovine cytochrome *c* oxidase subunits VIb and VIc, respectively, were prepared from mice immunized with the holoenzyme, as described (Taanman et al., 1993).

*Yeast Strains, Media, and Culture Conditions.* The *S. cerevisiae* wild-type strain JHRY1-2C$\alpha$ (*Mat*$\alpha$, leu2-3, leu2-122, ura3-52, his4-519, ade6, trp1) was the gift of Dr. T. H. Stevens (University of Oregon, Eugene, OR). *S. cerevisiae* strain J$\Delta$6a-1 (*Mat*$\alpha$, leu2-3, leu2-122, ura3-52, his4-519, ade6, trp1, cox13$\Delta$::LEU2) is a derivative of JHRY1-2C$\alpha$ in which the chromosomal copy of *COX13* (the gene coding for cytochrome *c* oxidase subunit VIa) has been replaced by the *LEU2* gene (Taanman & Capaldi, 1993). Yeast were routinely grown at 30 °C to the late log phase, in a 20-L fermentor (Model SA-30, Fermentation Design Inc.) with maximal aeration on 1% (w/v) Difco Bacto-yeast extract and 2% (w/v) Difco Bacto-peptone (YEP), supplemented with 1% (w/v) raffinose. Raffinose was chosen as the carbon source to prevent glucose repression (Lustig et al., 1982)

*Cytochrome c Oxidase Preparations.* Yeast cytochrome *c* oxidase was isolated by ion exchange chromatography using dodecyl maltoside as a solubilizing detergent, as described (Taanman & Capaldi, 1992), with the following modifications. Mitochondrial membranes were prepared from yeast cells resuspended in ice-cold resuspension buffer [50 mM Tris·HCl, pH 7.5, 400 mM mannitol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 $\mu$g/mL leupeptin, and 0.5 $\mu$g/mL pepstatin A], mixed with 0.5-mm glass beads, and homogenized in a Bead-Beater (Biospec Products) with six 1-min bursts at 3-min intervals (Jazwinski, 1990). After homogenization, the glass beads were allowed to settle and the cell slurry was collected. The glass beads were washed once with resuspension buffer to increase the yield. Next, cell debris was removed from the slurry by centrifugation at 3000*g* and 4 °C for 15 min. The pellet was washed once with resuspension buffer to increase the yield. Mitochondria were collected by centrifugation at 20 000*g* and 4 °C for 20 min. The mitochondrial pellet was washed four times, resuspended in a minimal volume of resuspension buffer, snap-frozen in liquid $N_2$, and stored at −80 °C until further use. Mitochondria were resuspended at a concentration of 30 mg of protein/mL in ice-cold resuspension buffer and centrifuged at 100 000*g* and 4 °C for 1 h. The mitochondrial pellet was resuspended at a concentration of 10 mg of protein/mL in ice-cold 50 mM Tris·HCl, pH 8.0, 1 mM $MgCl_2$, 8 mg/mL dodecyl maltoside, 1 mM PMSF, 0.5 $\mu$g/mL leupeptin, and 0.5 $\mu$g/mL pepstatin A and incubated at 4 °C for 30 min. Then, the mixture was centrifuged at 100 000*g* and 4 °C for 1.5 h. A 5 M solution of NaCl was added to the clear supernatant at a final concentration of 100 mM. Cytochrome *c* oxidase was isolated from the supernatant on a DEAE-Bio-Gel A column (2.5-cm diameter) equilibrated with 50 mM Tris·HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 0.02% (w/v) dodecyl maltoside, 1 mM PMSF, 0.5 $\mu$g/mL leupeptin, and 0.5 $\mu$g/mL pepstatin A, as described previously (Taanman & Capaldi, 1992). Peak fractions were stored in liquid $N_2$.

[1] Abbreviations: 2-$N_3$ATP, 2-azidoadenosine 5′-triphosphate; 8-$N_3$-ATP, 8-azidoadenosine 5′-triphosphate; TNP-ADP, 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate; TNP-ATP, 2′(or 3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidine difluoride); SDS, sodium dodecyl sulfate.

Bovine heart and kidney cytochrome *c* oxidases were purified according to the method of Capaldi and Hayashi (1972) and stored in small aliquots at −20 °C until use.

*Cytochrome c Oxidase Activity Measurements.* Concentrations of heme $aa_3$ were determined by air-oxidized *versus* sodium dithionite-reduced difference spectra recorded at room temperature on a Beckmann DU-7 spectrophotometer, using an extinction coefficient of 24.0 $mM^{-1}$ $cm^{-1}$ at 605 nm (Van Gelder, 1966). The steady-state activity of the dodecyl maltoside-solubilized cytochrome *c* oxidase (2 nM heme $aa_3$) was determined spectrophotometrically at 25 °C in 25 mM potassium phosphate, pH 6.2, 0.005% (w/v) dodecyl maltoside, and various concentrations of adenine nucleotides, as indicated in Results. After a preincubation of 8 min to ensure that binding equilibrium had been reached, the reaction was started by adding ferrocytochrome *c* to a final concentration of 50 μM. The initial rate of oxidation was determined by following the decrease in absorbance at 550 nm measured on an SLM Aminco DW-2000 spectrophotometer. Concentrations of ferrocytochrome *c* were determined using an extinction coefficient of 21.1 $mM^{-1}$ $cm^{-1}$ (Yonetani, 1965).

*Fluorescence Measurements.* The fluorescence emission of TNP-ATP and TNP-ADP was measured on an SLM Aminco DW-8000 spectrofluorometer. The samples were excited at 408 nm (slit width of 8 nm), and the emission spectra were recorded between 450 and 650 nm (slit width of 2–16 nm). The TNP-nucleotide titrations were performed in a 0.4-× 1-cm quartz cuvette (short path as excitation path) containing 400 μL of 110 nM cytochrome *c* oxidase dissolved in 10 mM K·HEPES, pH 7.4, and 0.005% (w/v) dodecyl maltoside. Additions of 400 μM or 3.2 mM TNP-nucleotide stock solutions were made in increments of 0.2–2.0 μL with a 1-μL Hamilton syringe to final concentrations of 0.2–26 μM, and the solution was mixed. After each addition, emission spectra were recorded when the emission at 534 nm had reached a stable plateau value. Parallel measurements under otherwise identical conditions were carried out in the absence of enzyme. All spectra were acquired in the photon counting mode as a ratio of the emission to excitation channels. The intensity of the emission light was adjusted by changing the width of the emission slits. After acquisition, spectra were corrected for background signals, dilution effects, and different slit widths. Spectra were also corrected for the inner filter effect by using a factor of $10^{-(0.4\times A_{408nm}\times 0.5)}$ to correct for attenuation of the exciting light and a factor of $10^{-(A_{\lambda nm}\times 0.5)}$ to correct for attenuation of the emission light. Application of these corrections to the spectra acquired in the absence of enzyme showed that the emission intensity of free TNP-nucleotides was proportional to their concentration. The concentrations of TNP-nucleotides were determined using an extinction coefficient of $2.64 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 408 nm (Hiratsuka & Uchida, 1973).

*Determination of Dissociation Constants.* Each spectrum, taken in the presence of the enzyme, is the sum of the spectrum emitted by the bound TNP-nucleotide and the spectrum emitted by the remaining free TNP-nucleotide:

$$S(\lambda) = c_T[x_B B(\lambda) + (1 - x_B)F(\lambda)] \quad (1)$$

where $S(\lambda)$ is the observed emission intensity, $B(\lambda)$ is the emission intensity of the bound TNP-nucleotide, $F(\lambda)$ is the emission intensity of the free TNP-nucleotide, $x_B$ is the fraction of bound TNP-nucleotide over the total, and $c_T$ is the total concentration of added TNP-nucleotide. In eq 1, $S(\lambda)$ and $F(\lambda)$ can be measured directly, and $B(\lambda)$ can be estimated (see below). Therefore, the fraction $x_B$ for each concentration $c_T$ is the parameter that optimizes the correspondence between the right-hand and left-hand sides of eq 1. The least-squares calculation gives

$$x_B = \frac{\sum S - c_T \sum F}{c_T \sum (B - F)} \quad (2)$$

where the symbol $\Sigma$ indicates the sum over a defined number of wavelength points of the indicated emission intensities. In the present work, 120 wavelength points were taken for each spectrum, from 510 to 630 nm.

The emission spectrum of the bound TNP-nucleotide, $B(\lambda)$, was assessed with an iterative procedure. First, the titrations of the mutant enzyme were analyzed, for which it was assumed that one single binding site was being titrated. For the first iteration it was assumed that, at the highest concentration point, the binding was at saturation. Therefore, $x_B$ is the concentration of the enzyme, and $B(\lambda)$ could be calculated from eq 1 and subsequently used to calculate $x_B$ throughout the titration. The data obtained in this way were fit by nonlinear least-squares analysis using the following equation:

$$x_B c_T = \frac{cE}{K_d + c} \quad (3)$$

where $c$ is the concentration of free TNP-nucleotide, $E$ is the concentration of enzyme, and $K_d$ the dissociation constant. $E$ and $K_d$ were chosen as fitting parameters. The best fitting parameters were used for the second iteration to calculate $x_B$ for the highest concentration point and, therefore, a new $B(\lambda)$ from eq 1. The new $x_B$ values were fit again with eq 2 and so on. Successive iteration steps caused the best fit value of $E$ to converge toward the actual enzyme concentration used in the titrations. The iteration procedure was stopped when the difference between the best fit value and the actual value was less than 10% of the actual value.

The same procedure was applied to the titrations of the wild-type enzyme. In this case, a first iteration sequence was performed by assuming a single binding site, as in the mutant. By this assumption, the resulting spectrum $B(\lambda)$ for each TNP-nucleotide was similar to the one obtained for the mutant enzyme, but its emission intensity was twice as much. A second type of iteration sequence for the wild type was performed by assuming two independent sites, one of which was common to both mutant and wild-type enzymes. Using the parameters found for the mutant, the contribution of the common site was subtracted for each point of the titration, and the iteration procedure was carried out for the corrected traces.

The calculations described were performed with the help of BASIC routines running on a personal computer.

*Photoaffinity Labeling, Protease Digestion, and Immunodetection.* In the photoaffinity labeling experiments, 26.5 μL of 4 μM yeast cytochrome *c* oxidase in 50 mM Tris·HCl, pH 8.0, 250 mM NaCl, and 0.01% (w/v) dodecyl maltoside was mixed with 1.5 μL of 400 μM [$\beta,\gamma$-$^{32}$P]-2-$N_3$ATP in the dark on ice. For bovine cytochrome *c* oxidase, 28 μL of 1.7 μM heart or kidney enzyme in 25 mM Tris·HCl, pH 7.6, and 0.05% (w/v) dodecyl maltoside was mixed with 0.5 μL of 400 μM [$\beta,\gamma$-$^{32}$P]-2-$N_3$ATP in the dark on ice. The ratio of enzyme to photoreactive ATP analogue was approximately 1:2, taking into account that under the conditions used only 40% of the ATP analogue population is in the photoreactive azido isomer form (Czarnecki, 1984). Mixtures were irradiated at 366 nm for 15 min on ice with a UV lamp (Model UVL-56, UVP Inc.) at a distance of 4 cm. For protease

digestion studies, trypsin was added to the irradiated samples at a ratio of 1:20 (trypsin/cytochrome *c* oxidase, w/w), and 1 M KCl was added at a concentration of 20 mM. Yeast cytochrome *c* oxidase was digested for 30 min at room temperature, whereas bovine cytochrome *c* oxidase was digested for 3 h at room temperature. The reaction was terminated by adding 1 M PMSF in acetone at a concentration of 1 mM. To analyze the reaction products, samples were dissociated at 37 °C in 4% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris·HCl, pH 6.8, and 12% (v/v) glycerol for 30 min. The dissociation mixture was supplemented with 2% (v/v) β-mercaptoethanol in the samples with yeast cytochrome *c* oxidase. Dissociated protein samples were resolved on SDS–polyacrylamide minigels (5 cm long) as described (Taanman & Capaldi, 1992). For autoradiography, gels were stained with Coomassie Brilliant Blue R (Taanman & Capaldi, 1992), dried, and exposed to Fuji RX X-ray film with an intensifying screen at −80 °C for up to 2 days. For immunodetection, gels were blotted onto PVDF membranes (Towbin et al., 1979), and cytochrome *c* oxidase subunits of interest were vizualized with antibodies as described previously (Taanman & Capaldi, 1993).

## RESULTS

*Effects of ATP and ADP on the Activity of Wild-Type and Mutant Cytochrome c Oxidase.* Previously, we described the isolation and initial characterization of the gene for subunit VIa in the yeast *Saccharomyces cerevisiae* (Taanman & Capaldi, 1993). The cytochrome *c* oxidase complex was fully assembled in the *null* strain lacking subunit VIa, but there were clear differences in the enzyme activity of this mutant compared with the wild-type parental strain when assayed in mitochondrial preparations. For example, at low ionic strength (25 mM potassium phosphate, pH 6.2), the cytochrome *c* oxidase activity of the *null* strain was higher than the activity of the wild-type strain. With increasing concentrations of ADP, the cytochrome *c* oxidase activity of the *null* and wild-type strains increased in parallel. Increasing concentrations of ATP, however, enhanced the cytochrome *c* oxidase activity of the *null* strain to a much higher extent than the activity of the wild-type strain. To study the binding of nucleotides and nucleotide derivatives to cytochrome *c* oxidase directly, it was necessary to purify the enzyme as described in Materials and Methods. The subunit composition of enzyme obtained from the *null* mutant and the wild-type strain proved to be the same when analyzed on highly resolving SDS–polyacrylamide gels, except that subunit VIa was missing from the enzyme isolated from the *null* strain (not shown). Spectrophotometric activity assays of dodecyl maltoside-solubilized cytochrome *c* oxidase were performed in the absence and presence of increasing amounts of ATP or ADP. The results, depicted in Figure 1, show that the effects of increasing concentrations of ATP or ADP on the activity of the isolated enzyme are very similar to those observed using detergent-solubilized mitochondria from both strains [cf Figure 8 in Taanman and Capaldi (1993)]. In the presence of 5 mM ATP, mutant cytochrome *c* oxidase is 2.7-fold more active than in the absence of ATP. This high degree of stimulation of the activity of the mutant enzyme by ATP, compared to the wild-type enzyme, suggests the binding of ATP to two sites: (1) an activating site on the core cytochrome *c* oxidase complex missing subunit VIa and (2) an inactivating site, involving subunit VIa, that prevents the increase in activity observed in the core complex. The dissociation constant of the activating ATP binding site present on the mutant enzyme

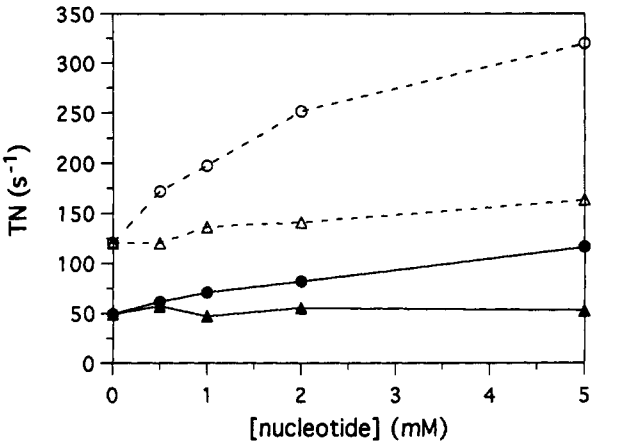


FIGURE 1: Effects of nucleotides on the activity of cytochrome *c* oxidase from different yeast strains. The turnover number (TN, moles of ferrocytochrome *c* oxidized per mole of heme $aa_3$ per second) of wild-type (JHRY1-2Cα) cytochrome *c* oxidase (solid line, closed symbols) and *null* mutant (JΔ6a-1) cytochrome *c* oxidase (dashed line, open symbols) was determined spectrophotometrically in 25 mM potassium phosphate, pH 6.2, 0.005% (w/v) dodecyl maltoside, and various concentrations of ATP (circles) or ADP (triangles), at 25 °C, in the presence of 50 μM ferrocytochrome *c*. Values are the mean of four separate determinations; the standard deviation was never more than 7% of the mean value.

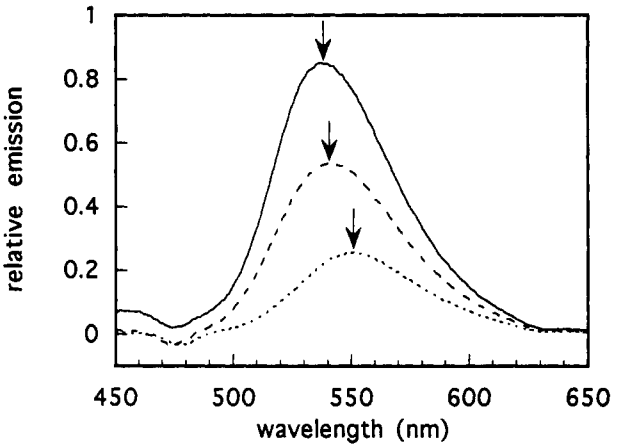


FIGURE 2: Interaction of TNP-ATP with cytochrome *c* oxidase from different yeast strains. Fluorescence spectra were recorded as indicated in Materials and Methods and corrected for background. Cytochrome *c* oxidases from wild-type (JHRY1-2Cα, solid line) and mutant (JΔ6a-1, dashed line) strains were both present at a concentration of 110 nM in 10 mM K-HEPES, pH 7.5, and 0.005% (w/v) dodecyl maltoside. TNP-ATP was present at a concentration of 3 μM. The dotted line represents the fluorescence emission of TNP-ATP without cytochrome *c* oxidase. Blue shifts of the emision maxima are indicated by arrows.

was estimated to be 2.8 mM, using nonlinear least-squares fit analysis of the data presented in Figure 1.

*Stoichiometric Binding of TNP-ATP and TNP-ADP to Wild-Type and Mutant Cytochrome c Oxidase.* The interactions of adenine nucleotides with cytochrome *c* oxidase were studied with the fluorescent nucleotides TNP-ATP and TNP-ADP. Measurements of the fluorescence emission of TNP-ATP added to a cuvette containing wild-type or mutant cytochrome *c* oxidase showed a marked increase in the emission intensity and a blue shift of the emission maximum ($\lambda_{max}$) over the fluorescence emission in the absence of cytochrome *c* oxidase (Figure 2). The enhancement of the emission intensity and the blue shift were more pronounced with the wild-type enzyme than with the mutant enzyme. The changes in spectral properties of TNP-ATP indicate that the nucleotide analogue binds to the protein and allowed us to perform

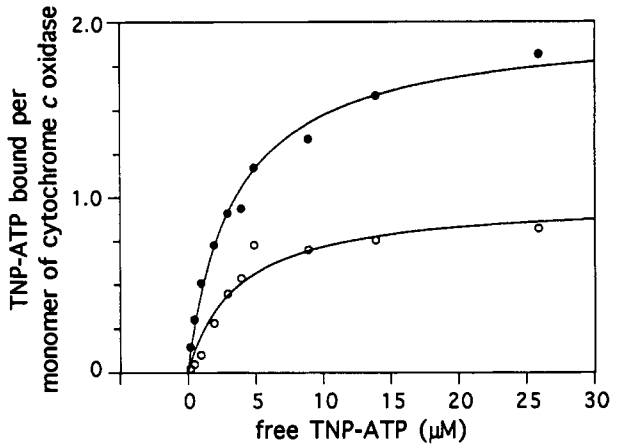


FIGURE 3: Binding curves of cytochrome c oxidase from different yeast strains with TNP-ATP. Wild-type (JHRY1-2Cα, closed circles) and mutant (JΔ6a-1, open circles) cytochrome c oxidases were titrated with TNP-ATP in 10 mM K·HEPES, pH 7.5, and 0.005% (w/v) dodecyl maltoside, and the fraction of bound TNP-ATP was assessed as described in Materials and Methods. The continuous lines are computer simulations of the stoichiometry using nonlinear least-squares fit analysis of the observed values.

equilibrium binding studies. Titrations of the wild-type and mutant cytochrome c oxidases with increasing concentrations of TNP-ATP were carried out and showed saturation at increasing concentrations of the fluorescent ATP analogue. However, the level of saturation for the mutant enzyme was one-half that for the wild-type enzyme. The data were used to estimate the amount of TNP-ATP bound per monomer of cytochrome c oxidase with the iterative procedure described in Materials and Methods. The binding curves of TNP-ATP with wild-type and mutant enzymes are shown in Figure 3. The most plausible interpretation of these data (see Materials and Methods) is that there are two binding sites for the nucleotide analogue on the wild-type enzyme and one binding site on the mutant enzyme. This result is additional strong evidence that one binding site for ATP is located on subunit VIa, the subunit that is missing from the mutant enzyme.

Parallel titrations were performed with TNP-ADP instead of TNP-ATP. These titrations gave very similar results. Upon interaction with cytochrome c oxidase, TNP-ADP exhibited an identical strong enhancement of the fluorescence and blue shift of the maximum. In addition, TNP-ADP bound at stoichiometries of near 1 per monomer of mutant cytochrome c oxidase and near 2 per monomer of wild-type cytochrome c oxidase (not shown). The apparent dissociation constants for the activating binding site on the mutant enzyme ($K_{d1}$) and the inactivating binding site involving subunit VIa ($K_{d2}$) were determined by a best fit of the data using eq 3. The results for both TNP-nucleotides are compiled in Table 1. The binding of TNP-ATP to mutant cytochrome c oxidase ($K_{d1}$ = 3.8 μM, Table 1) is much tighter than that of ATP determined from activity assays ($K_d$ = 2.8 mM, Figure 1), presumably because the presence of the trinitrophenyl group increases the affinity of the fluorescent ATP analogue for the nucleotide binding site on the enzyme. The dissociation constants for TNP-ATP and TNP-ADP are similar for both binding sites. This may be due to the contribution of the trinitrophenyl group to the affinity and is not necessarily true for ATP and ADP.

*Effects of TNP-ATP and TNP-ADP on the Activity of Wild-Type and Mutant Cytochrome c Oxidase.* On the basis of the binding studies, TNP-ATP should lead to the same marked stimulation of the activity of the mutant enzyme as was observed for ATP, but at a much lower concentration. To test this, the effects of TNP-ATP and TNP-ADP on the cytochrome c oxidase activity were studied under the same conditions used for ATP and ADP, but at a 1000-fold lower nucleotide concentration. The results for the TNP-adenine nucleotides, shown in Figure 4, are strikingly similar to the results obtained with adenine nucleotides shown in Figure 1. With increasing concentrations of TNP-ADP, the activities of both wild-type and mutant cytochrome c oxidase increase slightly. TNP-ATP has the same effect as TNP-ADP on the wild-type enzyme, but it enhances the activity of the mutant enzyme to a high degree. In the presence of 20 μM TNP-ATP, mutant cytochrome c oxidase is 2.6-fold more active than in the absence of the ATP analogue. The apparent dissociation constant of the TNP-ATP binding site on the mutant enzyme was estimated to be around 20 μM using nonlinear least-squares fit analysis of the data presented in Figure 4. This dissociation constant is 5-fold higher than those found in the fluorescence titrations (Table 1). The activity assays were performed in a 25 mM potassium phosphate buffer, pH 6.2, whereas the titrations were done in a 10 mM K·HEPES buffer, pH 7.5. We assume that buffer conditions are responsible for the minor differences in the estimated dissociation constants. The activity assays with TNP-ATP clearly demonstrate that this fluorescent ATP analogue causes the same effect as ATP on the activity of the mutant enzyme, but at a much lower concentration, indicating that the ATP moiety of the TNP-ATP interacts in the same manner as ATP with cytochrome c oxidase.

*Photoaffinity Labeling of Yeast Cytochrome c Oxidase with 2-$N_3$ATP.* The experiments described above argue for the presence of an ATP binding site on subunit VIa of yeast cytochrome c oxidase. To determine this more directly and in an effort to localize the second binding site indicated by the fluorescence titrations, we performed labeling studies with the photoreactive ATP analogue, 2-$N_3$ATP. Both wild-type and mutant yeast cytochrome c oxidase were mixed with radioactive 2-$N_3$ATP and exposed to ultraviolet light for covalent binding of the ATP analogue. Then, part of the samples were incubated with trypsin. Mild proteolytic digestion with trypsin removes the subunits VIa and VIb from the detergent-solubilized enzyme complex of beef (Ludwig et al., 1979; Zhang et al., 1988; Planques et al., 1989) and yeast,[2] but it does not remove any other subunits from the complex. Incorporation of the photoaffinity nucleotide analogue was localized by gel electrophoresis, followed by autoradiography of the gels as described in Materials and Methods. Typical results are presented in Figure 5. In wild-type enzyme, there was one major labeled band migrating just below the 14 000 marker. This radioactive band was absent from the *null* mutant missing subunit VIa, a strong argument that it represents 2-$N_3$ATP-labeled subunit VIa. The altered migration of the 2-$N_3$ATP-modified subunit VIa with respect to unmodified subunit is not surprising given the negative charges added. Further evidence that the radioactive band is 2-$N_3$ATP-modified subunit VIa and not subunit VI (which comigrates with the radioactivity) was obtained in the trypsin cleavage experiment. As shown by the immunoblots in Figure 5, trypsin digested subunit VIa and the modified form of this subunit, but not subunit VI.

The autoradiogram of Figure 5 shows a faint band migrating between the 14 000 and 6000 markers in the sample with

[2] S. D. Watts, J.-W. Taanman, and R. A. Capaldi, unpublished observations.

Table 1: Spectral Changes and Dissociation Constants ($K_d$) of TNP-Nucleotides Observed upon Interaction with Cytochrome *c* Oxidase from Different Yeast Strains

| yeast strain | nucleotide | $\lambda_{max}$ of free nucleotide (nm) | $\lambda_{max}$ of bound nucleotide (nm) | fluorescence increase at $\lambda_{max}$ upon binding (-fold) | $K_{d1}$[a] ($\mu$M) | $K_{d2}$[b] ($\mu$M) |
|---|---|---|---|---|---|---|
| JΔ6a-1 (*null*) | TNP-ATP | 550 | 536 | 55–60 | 3.8 | |
| JΔ6a-1 (*null*) | TNP-ADP | 550 | 536 | 100–110 | 3.7 | |
| JHRY1-2Cα | TNP-ATP | 550 | 536 | 55–60 | | 2.7 |
| JHRY1-2Cα | TNP-ADP | 550 | 536 | 100–110 | | 3.7 |

[a] Activating binding site present on the core cytochrome *c* oxidase complex missing subunit VIa. [b] Inactivating binding site involving cytochrome *c* oxidase subunit VIa.

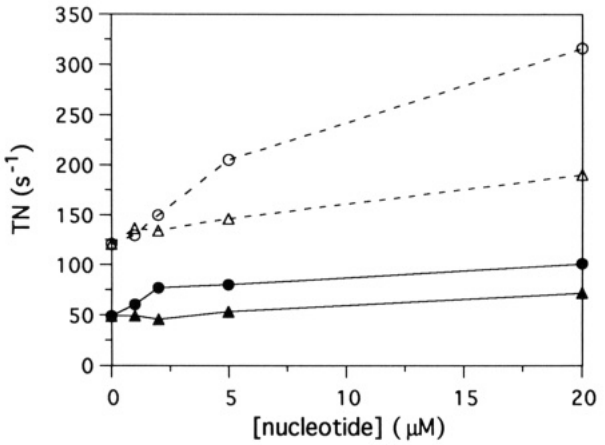


FIGURE 4: Effects of TNP-nucleotides on the activity of cytochrome *c* oxidase from different yeast strains. The turnover number (TN, moles of ferrocytochrome *c* oxidized per mole of heme $aa_3$ per second) of wild-type (JHRY1-2Cα) cytochrome *c* oxidase (solid line, closed symbols) and *null* mutant (JΔ6a-1) cytochrome *c* oxidase (dashed line, open symbols) was determined spectrophotometrically in 25 mM potassium phosphate, pH 6.2, 0.005% (w/v) dodecyl maltoside, and various concentrations of TNP-ATP (circles) or TNP-ADP (triangles), at 25 °C, in the presence of 50 $\mu$M ferrocytochrome *c*. Values are the mean of four separate determinations; the standard deviation was never more than 10% of the mean value.

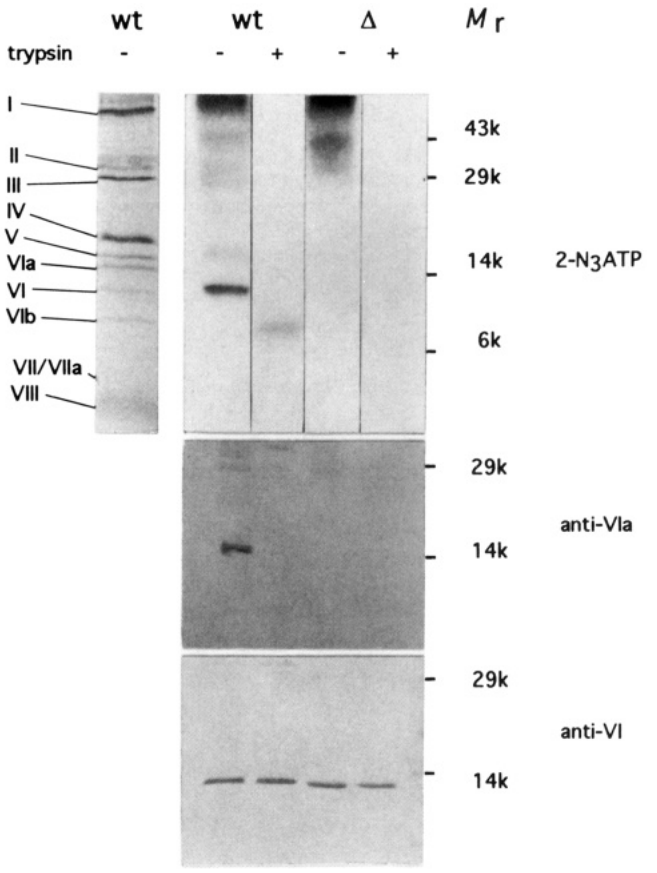


FIGURE 5: Photoaffinity labeling of cytochrome *c* oxidase from different yeast strains with 2-$N_3$ATP. The upper panel shows an autoradiogram of wild-type (wt, JHRY1-2Cα) and mutant (Δ, JΔ6a-1) cytochrome *c* oxidases reacted with radioactive 2-$N_3$ATP. After the reaction, samples were split in two, and one part was incubated with trypsin as indicated. The subunit pattern of wild-type cytochrome *c* oxidase, stained with Coomassie Brilliant Blue and silver (Taanman & Capaldi, 1992), is shown on the left. The lower two panels show immunoblots of the same samples visualized with monoclonal antibodies against subunits VIa and VI. The migration of molecular weight standards is indicated.

wild-type cytochrome *c* oxidase that has been incubated with trypsin. This band was not visible in samples incubated with trypsin for longer periods of time (not shown). Therefore, we conclude that this band is a transient degradation product of subunit VIa. A transient degradation product of subunit VIa has also been observed in detergent-solubilized bovine cytochrome *c* oxidase treated with trypsin (Zhang et al., 1988).

The only other major labeling products observed in Figure 5 are diffuse smears present in the upper part of the autoradiogram. These are not present in the samples that have been incubated with trypsin, indicating that they result from the labeling of minor high molecular weight impurities, e.g., $F_1$-ATPase and chaperonin 60,[3] present in the cytochrome *c* oxidase preparations. Treatment with trypsin removes these contaminants (Ludwig et al., 1979). As there are no other major bands of radioactivity visible on the autoradiogram, the second nucleotide binding site of cytochrome *c* oxidase, demonstrated by the fluorescence titrations with TNP-adenine nucleotides, is not labeled efficiently by 2-$N_3$ATP.

*Photoaffinity Labeling of Bovine Cytochrome c Oxidase with 2-$N_3$ATP.* In mammals, two isoforms of cytochrome *c* oxidase subunit VIa are expressed tissue specifically. To investigate whether an ATP binding site is present on these isoforms, we expanded the photoaffinity labeling studies to the enzyme from bovine heart and kidney. Heart contains the so-called heart isoform, VIa-H, whereas kidney contains the so-called liver isoform, VIa-L. The autoradiogram showed only one prominent band, migrating below the 14 000 marker, in both heart and kidney cytochrome *c* oxidase (Figure 6A). This region of the gel contains the subunits VIa, VIb, and VIc. Trypsin digestion of the mammalian enzyme, which cleaves subunit VIb completely and most of subunit VIa but not subunit VIc (see the immunoblots of Figure 6B), removed the labeled band in both the heart and liver enzymes, with the production of a transient degradation product with a relative molecular weight of 6000 visible on longer exposed autoradiograms. This cleavage pattern is that of VIa and not VIb (Zhang et al., 1988).

## DISCUSSION

Most metabolic pathways are controlled by key enzymes, which are in turn regulated by the levels of substrates, products,

[3] J.-W. Taanman and R. A. Capaldi, unpublished observations.

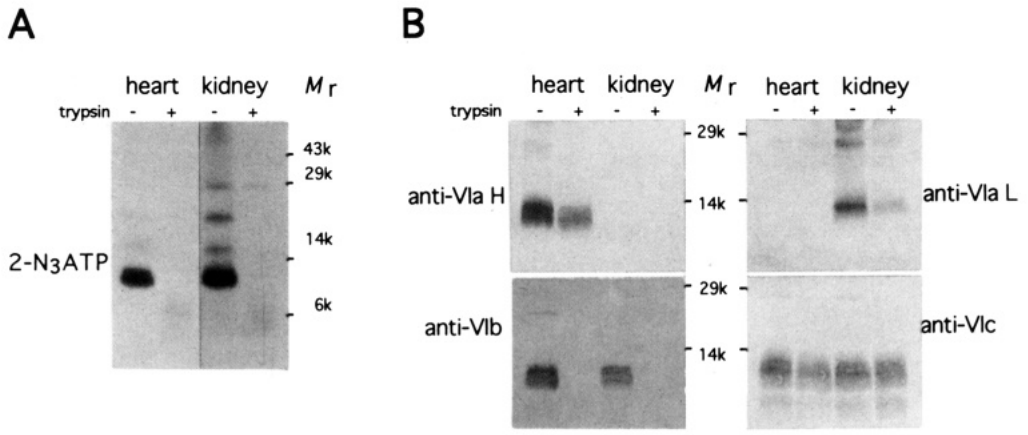


FIGURE 6: Photoaffinity labeling of bovine cytochrome *c* oxidase with 2-$N_3$ATP. (A) Autoradiogram of heart and kidney cytochrome *c* oxidases reacted with radioactive 2-$N_3$ATP. After the reaction, samples were split in two, and one part was incubated with trypsin as indicated. (B) Immunoblots of the same samples visualized with antibodies against subunits VIa-H, VIa-L, VIb, and VIc. The migration of molecular weight standards is indicated.

and other allosteric effectors. The hypothesis that cytochrome *c* oxidase, as the key enzyme of the respiratory chain, is regulated by adenine nucleotides as allosteric effectors (Kadenbach, 1986) is, in this respect, attractive. Effects of nucleotides on cytochrome *c* oxidase activity have been described repeatedly (Ferguson-Miller et al., 1976; Roberts & Hess, 1977; Osheroff et al., 1978; Hüther & Kadenbach, 1986, 1987, 1988; Malatesta et al., 1987; Bisson et al., 1987; Rigoulet et al., 1987; Hüther et al., 1988; Reimann et al., 1988; Antonini et al., 1988; Kadenbach et al., 1991; Anthony et al., 1993; Taanman & Capaldi, 1993; Rohdich & Kadenbach, 1993). Several of these studies have reported that the so-called high-affinity binding site for cytochrome *c* on the enzyme is converted to low affinity by millimolar concentrations of nucleotides (Ferguson-Miller et al., 1976; Roberts & Hess, 1977; Osheroff et al., 1978; Hüther & Kadenbach, 1986, 1987, 1988; Bisson et al., 1987; Hüther et al., 1988), an effect that can be explained at least in part by an increase in the ionic strength that leads to a similar conversion of the affinity for cytochrome *c* (Wilms et al., 1980; Brooks & Nicholls, 1982; Sinjorgo et al., 1986).

A complicating problem in most of these studies is that no distinction was made between the interaction of nucleotides with cytochrome *c* oxidase or with its substrate, cytochrome *c* (Ferguson-Miller et al., 1976; Roberts & Hess, 1977; Osheroff et al., 1978; Hüther & Kadenbach, 1986; Malatesta et al., 1987; Bisson et al., 1987; Rigoulet et al., 1987; Hüther et al., 1988; Reimann et al., 1988; Antonini et al., 1988). Specific binding sites for adenine nucleotides have been identified on cytochrome *c* (Margoliash et al., 1970; Stellwagen & Shulman, 1973; Margalit & Schejter, 1973; Kayushin & Ajipa, 1973; Corthésy & Wallace, 1986, 1988). The binding of ATP to cytochrome *c*, at millimolar concentrations of ATP, diminishes the electron flow in the respiratory chain (Craig & Wallace, 1991, 1993), thus mimicking claimed effects of adenine nucleotides on the kinetics of cytochrome *c* oxidase.

In an attempt to distinguish between an interaction of ATP with cytochrome *c* oxidase or with cytochrome *c*, Kadenbach and colleagues (Hüther & Kadenbach, 1986, 1988; Hüther et al., 1988) used 8-$N_3$ATP to covalently modify the enzyme complex. This modification of the oxidase was found to have effects on the enzyme activity similar to free ATP. However, the validity of these experiments is questionable because the yield of the cross-linked product with such an analogue is inevitably very low, and autoradiograms revealed that cytochrome *c* oxidase had been labeled in an unspecific manner (Hüther et al., 1988; Reimann et al., 1988).

As a different approach, Kadenbach's group has examined the effects of ADP and ATP entrapped in vesicles with reconstituted bovine heart cytochrome *c* oxidase and found an effect on the activity (Hüther & Kadenbach, 1987, 1988). This effect cannot be due to nucleotide interaction with cytochrome *c* because the substrate was added to the outside of the vesicles. Parallel studies with cytochrome *c* oxidase from *Paracoccus*, which lacks the nuclear-coded subunits present in mammals, did not reveal such an interaction (Hüther & Kadenbach, 1988). These results were interpreted by Kadenbach and colleagues to indicate the binding of adenine nucleotides with one of the nuclear-coded subunits on the matrix side of the mitochondrial inner membrane. In a subsequent study, distinct effects of intraliposomal ATP and ADP were found for bovine heart and liver cytochrome *c* oxidase (Kadenbach et al., 1991), suggesting the involvement of one of the three tissue specifically expressed subunits. By using a monoclonal antibody specific for subunits VIa-H and VIc, the effects of the adenine nucleotides could be related to an interaction with the matrix domain of subunit isoform VIa-H (Anthony et al., 1993). In the presence of micromolar concentrations of intraliposomal ATP, millimolar concentrations of intraliposomal ADP were found to enhance the activity of bovine heart cytochrome *c* oxidase, but only by 8% (Rohdich & Kadenbach, 1993). The physiological significance of the small increase in activity, at conditions present in energy-depleted mitochondria, seems doubtful.

In our studies of nucleotide binding to cytochrome *c* oxidase, we have used enzyme from yeast and we have employed wild-type enzyme, as well as a highly active preparation in which subunit VIa has been genetically removed. Activity assays with wild-type yeast cytochrome *c* oxidase showed a slight increase in activity with increasing amounts of ATP. Parallel assays with cytochrome *c* oxidase isolated from the *null* mutant missing subunit VIa, however, showed a more than 250% increase in activity in the presence of 5 mM ATP over the activity in the absence of ATP (Figure 1). Apparently, the interaction of ATP with subunit VIa in the wild-type enzyme prevents the enhancement of the activity observed in the mutant enzyme. In addition, the stimulation of the mutant enzyme implies the presence of a second ATP binding site on the enzyme. Interaction of ATP with this second site leads to an increase in the activity, as observed in the mutant enzyme. Two binding sites for TNP-ATP per monomer of yeast cytochrome *c* oxidase were demonstrated by a quantitative titration (Figure 3). A titration with the mutant enzyme revealed only one binding site, thus confirming that one

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Bovine VIa-H: (38) HSGH--RERPAFIP--------YHHLRIRTKPFSWGDGNHTFFHNPRVNPLPTGYEKP
                    | |   ||| |           | |||||:||| ||||||| |||| |||||||||
Bovine VIa-L: (36) KSHHGEEERPEFVA--------YPHLRIRSKPFPWGDGNHTLFHNPHVNPLPTGYEDE
                      | |                 |    |||||| |||| ||| || ||      :
Yeast VIa   : (65) EKEHAEHREHLKHVPDSEWPRDYEFMNIRSKPFFWGDGDKTLFWNPVVNRHIEHDD
```

FIGURE 7: Alignment of the intermembrane space domain of cytochrome *c* oxidase subunit VIa polypeptides. The C-terminus of the yeast polypeptide is compared with the bovine isoforms VIa-H and VIa-L. The putative ATP binding site is underlined. The numbers in parentheses indicate residue numbers. Hyphens in the sequence denote insertions that have been made to improve alignment. Identical amino acid residues are indicated by vertical lines; conserved replacements are indicated by colons.

nucleotide binding site is at least in part located on subunit VIa, as indicated by the activity assays. The apparent dissociation constant for TNP-ATP is 1000-fold lower than that for ATP. The stimulation of the mutant enzyme with millimolar concentrations of ATP can also be achieved with micromolar concentrations of TNP-ATP (Figure 4). This fact clearly demonstrates that the stimulation is not caused by the ionic strength but by ATP itself. We found similar dissociation constants for TNP-ATP and TNP-ADP (Table 1), yet these adenine nucleotides have a different effect on the activity of the mutant enzyme (Figure 4). This indicates that the difference in effect is not caused by a difference in affinity for these adenine nucleotide analogues. In a preceding study, we found that potassium pyrophosphate had an effect on the cytochrome *c* oxidase activity of detergent-solubilized wild-type and mutant mitochondria similar to that of ADP, whereas GTP had the same effect as ATP (Taanman & Capaldi, 1993). Apparently, the triphosphate moiety of nucleotides is the prime determinant of the stimulation of the mutant enzyme. Reimann and Kadenbach (1992) have demonstrated two ATP binding sites per monomer of bovine cytochrome *c* oxidase by quantitative titration with TNP-ATP; however, the correlation between binding and activity was not examined.

Other groups have used 8-$N_3$ATP to investigate whether subunits of cytochrome *c* oxidase are involved in ATP binding. In one study, labeling was found for bovine cytochrome *c* oxidase in subunit IV along with subunits VIIa, VIIb, VIIc, and VIII (not resolved on the gel system used; Montecucco et al., 1986). However, as discussed earlier, Kadenbach and colleagues were unable to repeat this relatively specific labeling, and instead labeled almost every subunit of the enzyme complex (Hüther et al., 1988; Reimann et al., 1988). We have used low concentrations of 2-$N_3$ATP and not 8-$N_3$ATP in the labeling experiments because the 2-substituted analogue, similar to the natural ATP, possesses a preferred *anti* conformation of the purine with respect to the ribose, whereas 8-$N_3$ATP possesses a preferred *syn* conformation (Czarnecki et al., 1982). Covalent photolabeling of yeast cytochrome *c* oxidase with radioactive 2-$N_3$ATP confirmed the presence of an ATP binding site on subunit VIa (Figure 5). The labeling of both isoforms of bovine cytochrome *c* oxidase subunit VIa (Figure 6) indicates that the ATP binding site is conserved in the subunit from different species, as well as in different isoforms. The photolabeling experiments did not reveal the nature of the second ATP binding site demonstrated with the TNP-ATP titrations. Apparently, the photoreactive azido group in the adenine ring does not allow 2-$N_3$ATP to bind to this second site.

The C-terminal part of subunit VIa, which is located in the intermembrane space, is much more conserved than the N-terminal part (Taanman & Capaldi, 1993), suggesting that the common ATP binding site is located on the C-terminal part of the polypeptide. A comparison of the sequence of this region with amino acid sequences of ATP binding proteins leads us to suggest that the sequence GDGXX[T/S] (where X is any amino acid residue), a sequence invariant in all known subunits VIa of cytochrome *c* oxidase (Figure 7; Ewart et al., 1991), contributes to the ATP binding site in the subunit. The motif is also conserved in the chaperonin 60 family and has been suggested to be related to the binding motif of the $\beta$-phosphate of ATP found in cyclic AMP-dependent kinase family proteins (Lewis et al., 1992; Kubola et al., 1994). The C-terminal sequence of this motif in the yeast subunit VIa ($GDGD\underline{KTX_6V}$) matches the C-terminal part of the homology A sequence [$\underline{GX_4GK}[\underline{T}/S]\underline{X_6}[\underline{V}/I]$; Walker et al., 1982], which is known to be diagnostic of nucleotide binding proteins. If the GDGXXT motif is indeed involved in ATP binding, then the ATP binding domain of subunit VIa will be in the intermembrane space and not on the matrix sides as suggested by Kadenbach's group (Anthony et al., 1993). The binding of ATP may result in large conformational changes, as observed in chaperonins upon ATP binding, and inhibit the catalytic activity of cytochrome *c* oxidase, e.g., by decreasing the affinity for cytochrome *c*. To investigate these possibilities, we are currently generating site-directed mutations in the putative ATP binding domain in the C-terminal part of subunit VIa.

On the basis of our studies with the yeast enzyme, subunit VIa of cytochrome *c* oxidase acts as an inhibitor. In the absence of ATP, the mutant enzyme missing subunit VIa was more active than the wild-type enzyme. Moreover, as discussed earlier, interaction of ATP with subunit VIa prevented the stimulation of the activity observed in the mutant enzyme. The kinetic effects of ATP on the mutant enzyme are obtained at physiological concentrations of ATP (Schwenke et al., 1981). Our observations support a regulatory role for subunit VIa in which this subunit monitors the concentration of ATP in the intermembrane space and inhibits the enzyme activity at physiologically high concentrations of ATP. This is very different from the conclusions drawn by Kadenbach and colleagues on the basis of their work with the mammalian enzyme (Anthony et al., 1993; Reimann et al., 1993), which can be explained in part if these workers were examining the combined effects of two sites on the oxidase. By using the yeast enzyme and taking advantage of the *null* mutant in subunit VIa, we are able to differentiate the effects of nucleotide binding at the two sites.

Ongoing experiments, using novel ATP analogues for labeling studies, are aimed at localizing and further characterizing the activating binding site for nucleotides on the enzyme. In particular, it is important to know the sidedness of this activating binding site. If it is at the matrix side, this would allow the oxidase to compare the nucleotide conditions on both sides of the mitochondrial inner membrane and respond accordingly. In response, the enzyme may increase or decrease electron transfer rates and possibly decouple electron transfer from proton translocation through the enzyme under conditions of high ATP to allow continued electron transfer, concomitant oxidation of NADH, and therefore, continued Krebs cycle activity.

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