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Research Article

The ratio between the fast and slow forms of bovine cytochrome c oxidase is changed by cholate or nucleotides bound to the cholate-binding site close to the cytochrome a_3/Cu_B binuclear centre

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Abstract. We determined the fraction of 'slow' and 'fast' conformations of bovine cytochrome c oxidase, following the kinetics of cyanide binding to the oxidized enzyme. We investigated whether treatment of heart mitochondrial particles with different commercially available types of cholate (standard and ultra-pure) can affect the fraction of cytochrome c oxidase in the two states. Compared to standard cholate, the use of ultra-pure cholate for solubilization of heart mitochondrial particles significantly increased the fraction of the fast enzyme. Complete homogeneity (~ 100% fast) was observed when cytochrome c oxidase was solubilized with ultra-pure cholate from heart mitochondrial particles pre-equilibrated with AMP; equilibration with ADP yielded a much smaller fraction of fast enzyme (~ 35%). These observations are discussed on the basis of the structural relationships between the known cholate-binding site and the binuclear cytochrome a_3-Cu_B site: variation in the occupancy of this binding site with cholate or nucleotides may modify reactivity of the oxidized binuclear centre towards cyanide.

Key words. Mitochondrial particles; cytochrome oxidase; fast and slow conformation; cyanide binding; nucleotides; cholate; structure.

Cytochrome c oxidase (CcOX) is the terminal acceptor of the respiratory chain, located in the inner mitochondrial membrane of eucaryotes or in the bacterial plasma membrane. CcOX catalyses the reduction of dioxygen to water, using the electrons donated by ferrocytochrome c [1]. The free energy gain associated with the reaction sustains the transmembrane proton electrochemical gradient required for ATP synthesis. The crystal structures of CcOX from beef heart and *Paracoccus denitrificans* have been solved [2–5]. The O_2-binding site was confirmed to be a bimetallic centre, containing the so-called haem a_3-Cu_B site. The same site binds several Fe-haem/copper ligands, such as cyanide, carbon monoxide, azide and nitric oxide, which inhibit cell respiration with different efficiency and mechanisms [6–9]. The kinetics of cyanide binding to the oxidized state of purified bovine CcOX depend strongly on the enzyme history. This led to the definition of the so-called 'fast' and 'slow' cyanide-binding forms [10–14], the former reacting homogeneously and more rapidly ($k_{on} = 2\ M^{-1}\ s^{-1}$) than the latter which is also heterogeneous [see ref. 10 for a review]. It was apparent from

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early work [6], and subsequently extensively analysed [15], that the reaction of cyanide with some CcOX turnover intermediate is faster by several orders of magnitude ($k_{on} > 10^5$ M^{-1} s^{-1}).

The adenine nucleotides have been reported to regulate both the electron transfer and the H^+ pumping activity of the bovine enzyme [16–22]. Up to ten binding sites for the adenine nucleotides have been identified by equilibrium dialysis experiments with the purified enzyme [20]; all these sites may be partly or totally occupied by molecules of cholate [3], a detergent used to extract the enzyme from the membrane. Binding of ATP has been proposed to induce inhibition of CcOX due to allosteric interaction with subunit IV [20, 21]; ADP binding triggers functional effects opposite to ATP, inducing also a significant spectral change in the enzyme in the γ-band region [20]. Two cholate molecules, visible in the structure published by Tsukihara et al. [3], will be referred to here as cholate-1 (Ch-1) and cholate-2 (Ch-2). The Ch-1 molecule is close to the binuclear site (see Discussion), and through this site is connected to the hydrophobic pool where some of the structured phospholipids are located [3].

The stability and catalytic efficiency of CcOX are strongly affected by both detergent type and concentration [23, 24]. In this paper, we show that not only detergent but also equilibration of the mitochondrial particles with nucleotides prior to solubilization changes the reactivity of the oxidized enzyme towards cyanide, and thus the fast-to-slow ratio: employing AMP and (ultra pure) cholate stabilizes the fast state, while ADP stabilizes the slow state.

Materials and methods

Materials. Ascorbate, N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), horse heart cytochrome c and analytical grade sodium cholate were purchased from Sigma (St. Louis, Mo.), and used without further purification. Dodecyl-β-D-maltoside (lauryl-maltoside) was from Biomol (Hamburg, Germany); ultra-pure sodium cholate from Dojindo (Kumamoto, Japan). In this paper, sodium cholate from Sigma is referred to as standard cholate ($\sim$98% pure), sodium cholate from Dojindo as ultra-pure cholate ($>$98% pure).

Preparation of CcOX. CcOX was in all cases purified from bovine heart in the presence of 3% (w/v) cholate, according to Yoshikawa et al. [25], except that the enzyme was suspended in a final buffer containing 0.1% dodecyl-β-D-maltoside instead of Tween 20 [preparation (prep) A]. Some modifications to this procedure are summarized in table 1, together with some relevant properties of the purified enzyme: (i) prep B was carried out as prep A except that the mitochondrial fraction was equilibrated with 5 mM AMP prior to the solubilization with standard cholate; (ii) in prep C, ultra-pure cholate was used instead of standard cholate during solubilization, and mitochondrial particles were equilibrated with either (iii) 5 mM ADP (prep D) or (iv) 5 mM AMP (prep E). Equilibration of the mitochondrial fraction with nucleotides always started 30 min before solubilization. Whenever nucleotides (AMP or ADP) were pre-equilibrated with mitochondrial particles, they were also maintained at the same concentration (5 mM) throughout all the subsequent purification steps, storage and measurements (unless stated otherwise). After preparation, the purified enzyme was either used immediately or stored at -70 °C until use. The enzyme concentration was determined using $\Delta\varepsilon_{444(red\text{-}ox)} = 156$ mM^{-1} cm^{-1} and is expressed as a functional monomeric unit (aa_3 f.u.).

CcOX activity. The activity of CcOX (2.5 nM f.u., table 1) was measured either spectrophotometrically or polarographically, in 20 mM Hepes-KOH pH 7.0 containing 70 mM KCl and 0.1% dodecyl-β-D-maltoside. In the spectrophotometric assay, the oxidation of ferrocytochrome c (20 μM) was followed at 550 nm by means of a double-beam spectrophotometer (Jasco, V-

Table 1. Properties of bovine cytochrome c oxidase prepared with standard or ultra-pure cholate

Enzyme preparation	Cholate	Soret λ max (nm)	Nucleotide before solubilization*	Nucleotide before the assay‡	Cyanide binding (% fast)	Activity (s^{-1})
A	standard	419	–	–	46	190
B	standard	422	AMP (5 mM)	–	85	213
B_1	standard	422	AMP (5 mM)	AMP (2.5mM) + ADP (2.5 mM)	49	174
C	ultra-pure	423	–	–	73	251
D	ultra-pure	418	ADP (5 mM)	–	37	190
D_1	ultra-pure	418	ADP (5 mM)	AMP (2.5mM) + ADP (2.5 mM)	46	175
E	ultra-pure	422	AMP (5 mM)	–	100	245

Assay conditions are given in Materials and methods.
* Nucleotide pre-equilibrated with mitochondria prior to the addition of cholate.
‡ Nucleotide added to cytochrome c oxidase 30 min before cyanide-binding assay.

570) and initial rates of cytochrome c oxidation were calculated. In the polarographic assay, O_2 consumption sustained by 10 mM sodium ascorbate, 0.75 mM TMPD and 20 μM horse heart cytochrome c, was monitored using a Clark-type electrode.

Cyanide binding to CcOX. Binding of cyanide to the oxidized enzyme was measured using a spectrophotometer (HP-8453) equipped with a photodiode array detector, and a thermostated 1-cm-light-path cuvette. The absorbance changes, induced upon cyanide binding to cytochrome a_3^{3+}, were followed at room temperature at 432 nm in 100 mM sodium phosphate pH 7.4 containing 0.5% lauryl-maltoside. The observed time courses were fitted to two exponential decays (unless otherwise stated) and the fraction of the fast and slow oxidase calculated from the relative amplitudes of the two phases.

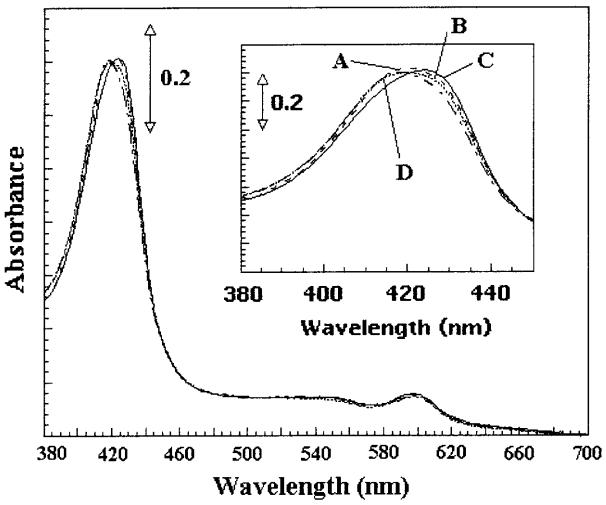

Figure 1. Optical spectra of CcOX purified from beef heart mitochondrial particles. Beef heart mitochondrial particles, were (i) solubilized with standard cholate without (prep A), or with pre-equilibration with AMP (prep B), (ii) solubilized with ultra-pure cholate without (prep C) or with pre-equilibration with ADP (prep D). The spectra were collected using an HP-8453 spectrophotometer (1-cm light path), in the absence (prep A and C) and presence (prep B and D) of the given nucleotide at 5 mM. CcOX, 4.5 μM (aa_3 functional units). Buffer: Hepes KOH, 0.1 M, pH 7.0; temperature 20 °C. *Inset*: magnification of the Soret region; peak position at ~418 nm, for prep A (standard cholate) and D (ultra-pure cholate + ADP), or at ~422 nm, for prep B (standard cholate + AMP) and C (ultra-pure cholate).

Results

Table 1 summarizes the spectral features and some of properties of the CcOX preparations, purified using two different cholates and in the presence/absence of AMP or ADP. The absorbance spectra of CcOX purified using standard and ultra-pure cholate, with or without pre-equilibration with AMP or ADP, were collected. As shown in figure 1, the position of the Soret peak differed: when purification is carried out using standard cholate or in the presence of ADP, the maximum is centred at ~418 nm, while it is red-shifted to ~422 nm when ultra-pure cholate is used, or the preparation is carried out in the presence of AMP (see table 1).

Binding of cyanide to the oxidized CcOX at room temperature is associated with a well-characterized spectral change, with an increase of absorbance at 432 nm. The time scale of the absorbance change (whether a few or several hundred minutes) and the kinetics (mono- or multiphasic) varies from preparation to preparation, and depends on the detergent used and on the presence of AMP or ADP, particularly when added at an early stage of purification before membrane solubilization (see Materials and methods).

The time courses of cyanide binding are shown in figure 2. When the enzyme was purified with ultra-pure cholate (prep C), cyanide binding was slow (minutes) and biphasic, with a predominant fast component (~73%), followed by a slower one that was completed within 2 h (prep C; fig. 2A). The same reaction, performed using the enzyme solubilized with standard cholate (prep A, fig. 2A), is also clearly biphasic, but the fast phase accounted for less than 50% of the overall change. When the mitochondrial fraction was pre-equilibrated with ADP (5 mM) prior to solubilization with ultra-pure cholate, cyanide binding was predominantly slow (prep D; fig. 2B), which suggests that the presence of ADP prior to solubilization may convert some of the enzyme from fast to slow. The opposite effect was elicited by AMP (prep B and E; fig. 2); in this case the spectrum had a maximum at ~422 nm (table 1) and was predominantly ($\geq$ 75%) in the fast cyanide-binding state. Homogeneity (~100% fast) was approached when both ultra-pure cholate and AMP (5 mM) were present during purification (Prep E). The effect of standard cholate in enhancing the fraction of the slow-reacting component (see above) could be partially compensated by pre-incubating the mitochondrial particles with AMP (5 mM), the slow component going from 46 to 15%.

Addition of AMP and ADP in equimolar amounts (2.5 mM) and just before the cyanide-binding assay always induced approximately equal amounts of the fast and slow state (table 1, enzyme treatment B_1, D_1). This finding was not further investigated, since the effect(s) of nucleotides added just before the assay are likely to involve interactions between the enzyme and the effectors (detergent and nucleotides) far from equilibrium. Enzyme activity (table 1) in the presence of bulk nucleotides was lower, probably based on partial masking of

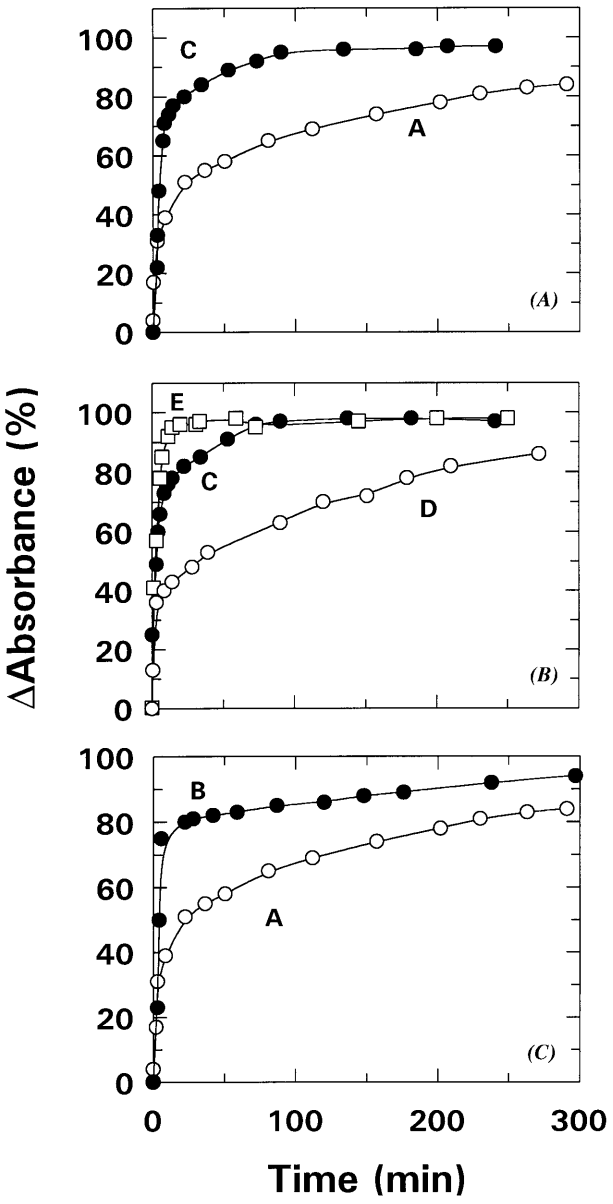

Figure 2. Effect of cholate and nucleotides on cyanide binding to oxidized CcOX. Time courses of cyanide binding to the oxidized enzyme (2.5 μM, aa_3 functional units). The reaction was carried out in Hepes KOH, 0.1 M pH 7.0, and followed at 432 nm after addition of 2 mM cyanide. Temperature 20 °C. (*A*) The enzyme solubilized with ultra-pure cholate is mostly 'fast' (prep C, ●), in contrast to that solubilized with standard cholate (prep A, ○). (*B*) Effect of addition of either 5 mM AMP (prep E, □) or 5 mM ADP (prep D, ○), prior to solubilization of mitochondria by ultra-pure cholate, compared to control (prep C, ●). When AMP was added prior to solubilization with ultra-pure cholate, the enzyme was completely in the 'fast' form, while the presence of ADP yielded a large fraction of the 'slow' form. (*C*) Effect of addition of 5 mM AMP (prep B, ●) prior to solubilization of mitochondria by standard cholate, compared to control (prep A, ○). Addition of AMP prior to solubilization elicits the 'fast' form.

the electrostatic interactions between reduced cytochrome c and CcOX. As anticipated by Tsukihara et al. [2], CcOX purified with ultra-pure cholate is characterized by a turnover number (TN) significantly higher than the enzyme purified with standard cholate: in good agreement with Tsukihara et al. [2], the TNs determined independently were 251 and 190 s^{-1}, respectively. Prep E (mitochondrial particles purified in the presence of AMP and using ultra-pure cholate) had a TN (251 s^{-1}) comparable to that of prep C.

Discussion

The present study has shown that the purity of the cholate used for mitochondrial particle solubilization and their pre-equilibration with AMP or ADP affect the reactivity of the binuclear site of purified CcOX towards cyanide, leading to different fractions of fast and slow enzyme [11]. As shown by Tsukihara et al. [3], the structural similarity between nucleotides and cholate suggests that they can compete for the same binding sites during solubilization of mitochondrial particles. In fact, bound cholate has been detected by radioactive labelling [20], and directly located in the crystal structure, whose formation depends critically on the detergent used and on cholate purity [3]. Dissociation of cholate from the purified bovine enzyme is very slow (hours) and, consequently, the onset of the functional effects observed on addition of nucleotides to the enzyme isolated in the presence of cholate is also slow [20, 21]. During the experiments designed to investigate the role of AMP and ADP on cyanide binding, to minimize interference by bound cholate, we equilibrated the mitochondrial particles with a high concentration (5 mM) of nucleotides before solubilization, and maintained this concentration during the subsequent purification procedure. This approach, which to our knowledge has never been attempted before, allowed us to investigate the effects of a bound nucleotide on the reactivity of the oxidized binuclear site in CcOX.

The model in figure 3 from the coordinates of Tsukihara et al. [2, 3] shows how ADP may be oriented in the cholate-binding site, Ch-1, close to the ligand-binding site of the enzyme. Ch-2 is omitted because it binds, relatively far away, on the surface of subunit VIa [3]. In figure 3, the binuclear site of one monomer is shown surrounded by the three core subunits (I, II and III) to illustrate that helix VIII of subunit I and helix III of subunit III provide the residues, namely Thr301 of subunit I, and Trp99 and His103 of subunit III, hydrogen-bonded to the Ch-1 molecule exposed to the cytosolic surface of the protein [3]. As shown in the figure, the maximum distance from ADP or these residues, and

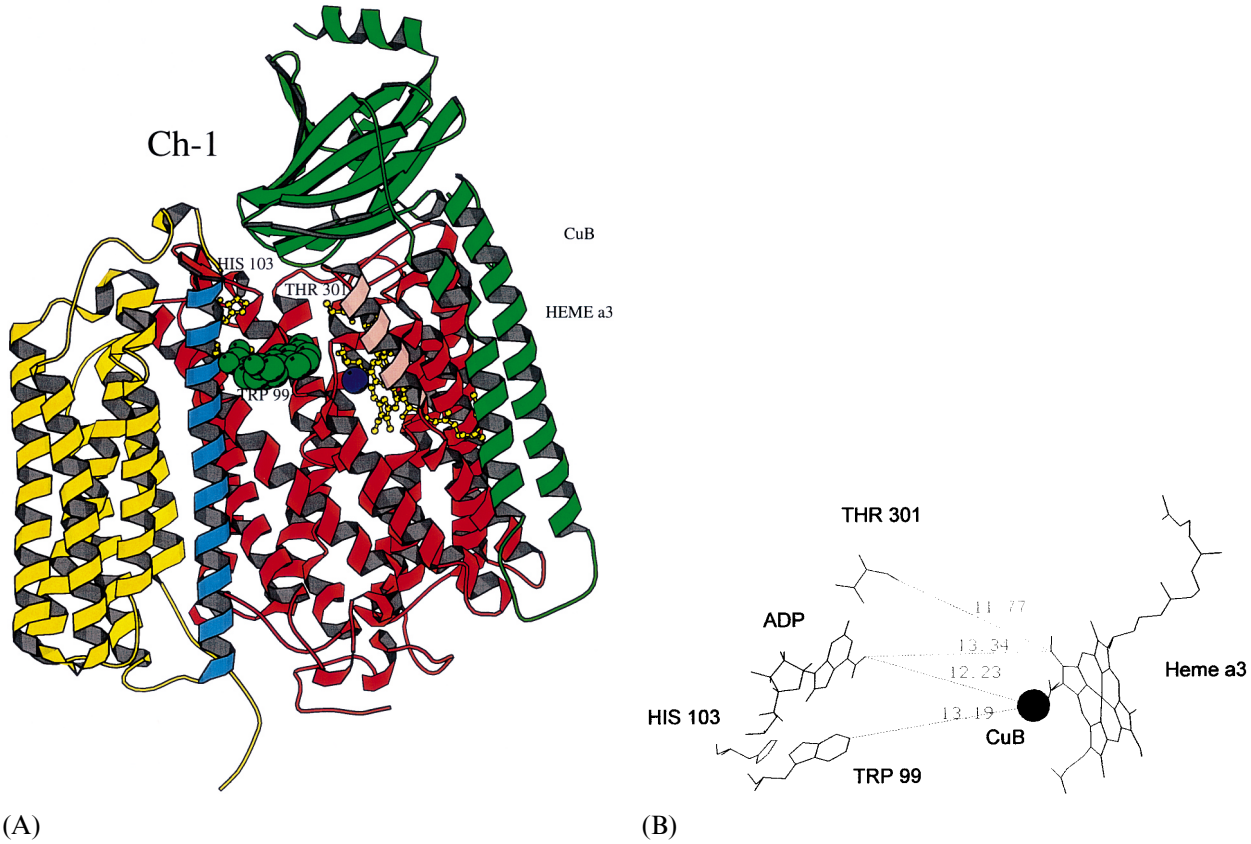

Figure 3. Structure of the cholate-binding site (Ch-1) and relationships with the active site of CcOX. (*A*) View (from the side) of the (core) subunit III (yellow), I (red) and II (green); helices VIII and III of subunit I and III are pink and blue, respectively. Orientation of the structure is chosen to show that the haem a_3-Cu_B site (haem, yellow sticks; Cu, blue ball) is in close proximity to the site hosting the Ch-1 molecule; a molecule of ADP (green balls) has been oriented in this site, as described in Tsukihara et al. [3], hydrogen-bonded to some amino acid residues (labelled, yellow sticks). The picture was generated using MOLSCRIPT [26]; coordinates from the structure no. 1OCC, Brookhaven Protein Data Bank, deposited by Tsukihara et al. [2, 3]. (*B*) Distance between ADP and the haem a_3 edge/Cu_B together with the distance between threonine 301 of subunit I and the haem a_3 edge, and tryptophan 99 of subunit III and Cu_B (calculated using Swisse Pdb.Viewer v. 3.51).

either Cu_B or haem a_3 (edge) in the active site, is from 11 to 13 Å (see fig. 3): we believe that this relatively short distance is compatible with cross-talk between haem a_3 and Cu_B on the one hand and the (bound) nucleotide or nucleotide-mimicking molecules, on the other. The overall size of an adenosine nucleotide is not inconsistent with this hypothesis.

We propose, therefore, that the extent and type of occupancy of the Ch1-binding site affects the geometry of the binuclear site with an effect on the kinetics of cyanide binding. This conclusion is suggested by the finding that both nucleotides and cholate control the relative population of the fast and slow cyanide-binding forms of the enzyme. The different perturbation of the active site seen with ADP compared to AMP (table 1) may be due to the additional negative charge of the phosphate introduced in the nearby Ch-1 site, and the fact that this observation is evident in the binding of an anion (CN^-) to the ferric binuclear centre is not inconsistent with an electrostatic effect. In agreement with this hypothesis, the absorption spectrum of the enzyme purified with ultra-pure cholate has the same Soret maximum ($\sim$ 422 nm) as that equilibrated with AMP, while that purified with standard cholate has the same maximum ($\sim$ 418 nm) as the ADP-equilibrated enzyme. In the absence of analytical characterization of the two cholate specimens, we may only assume that the $\sim$ 2% impurities of the standard cholate include an unknown contaminant able to mimick the ADP effect.

In conclusion, the procedure of pre-equilibrating the mitochondrial particles with nucleotides yielded the result that AMP and ADP have opposite effects on the cyanide-binding kinetics, and standard cholate may mimic ADP. Thus, when ADP is added prior to the solubilization with ultra-pure cholate, oxidized CcOX is mostly in the slow form, while mitochondrial equilibra-

tion with AMP stabilizes the fast form, even when standard cholate is used during solubilization. The combination of ultra-pure cholate solubilization and mitochondrial equilibration with AMP yields a homogeneous preparation of fast enzyme ($\sim$ 100%), suggesting that the two effects are to some extent additive. The results presented above suggest that the Ch-1 binding site of CcOX, in the presence of standard cholate (containing contaminant X), AMP or ADP, is coupled to cyanide reactivity (fast or slow) according to the following schematic view:

AMP;cholate $\rightleftarrows$ Ch-1 site $\rightleftarrows$ ADP;X

fast $\rightleftharpoons$ slow

Here, the fast and slow population ratio is shifted by the different molecules bound to the Ch-1 site.

Interestingly, CcOX activity also seems sensivite to the occupancy of the cholate-binding site, as the highest turnover number is observed when the enzyme preparation is carried out with ultra-pure cholate, and in the presence of AMP. This finding suggests that the occupancy by cholate or nucleotides (AMP, ADP) of the cholate-binding site(s) affects not only cyanide binding, i.e. accessibility of ligands to the oxidized binuclear site, but also the turnover number via activation of the rate-limiting step in catalysis, a phenomenon that remains to be elucidated.

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