Zinc Ions Inhibit the Qp Center of Bovine Heart Mitochondrial bc1 Complex by Blocking a Protonatable Group*

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Bovine heart bc1 complex is reversibly inhibited by zinc ions with an inhibition constant Kι of 10−7 M at pH 7.0. Binding of zinc is at least a factor of 10 tighter than binding of any other metal ion tested. Essentially complete inhibition of ubiquihydroquinone:cytochrome c oxidoreductase activity is observed at concentrations of [Zn2+] > 5 μM. Zinc does not affect the Kι for the substrates, ubiquihydroquinone or cytochrome c, but zinc inhibits reduction of the cytochromes by ubiquihydroquinone through the Qp center. A radioactive binding assay using 109Zn revealed one high affinity binding site per bc1 complex with Kι = 10−7 M at pH = 7.0 and 3–4 additional low affinity binding sites (Kι > 2 × 10−6 M). Zinc binding does not depend on the redox state of the high potential chain (iron-sulfur protein and cytochrome c). Zinc binds 3 times tighter to Fe-S-depleted bc1 complex indicating that the zinc binding site is not on the “Rieske” iron-sulfur protein in contrast to a recent report by Lorusso et al. (Lorusso, M., Cocco, T., Sardanella, A. M., Minuto, M., Bonomi, F., and Papa, S. (1991) Eur. J. Biochem. 197, 555-561). Zinc binds to a site which has the same affinity for zinc as for protons. We conclude that the zinc binding site is close to a protonatable group of the bc1 complex with pKa = 7.2 which has not been identified previously. We propose that this group is part of the proton channel at the hydroquinone oxidation center of the bc1 complex.

Inhibitors have been indispensable tools for elucidating the reactions of the ubiquitous bc1 complexes (for a review, see Ref. 1). Almost all of these inhibitors are aromatic organic compounds where at least part of the structure has some structural relationship to the substrate, ubiquihydroquinone. The single notable exception are zinc ions which have first been reported by Skulachev et al. (2) to inhibit mitochondrial respiration in micromolar concentrations. Subsequent studies (3–5) have established that the primary site of zinc inhibition in bovine heart mitochondria is the bc1 complex. The knowledge about the bc1 complex has largely increased over the past 20 years, including the structure of the redox centers and the peptide composition of the mammalian 11-subunit complex. The “Q-cycle” mechanism has been established for the sequence of electron and proton transfers (see Ref. 6). In the light of this body of information, we have reinvestigated inhibition by zinc to obtain information about the specific interaction between zinc ions and the bc1 complex and to use this information to get insight into mechanistic details of the electron and proton transfer reactions.

**MATERIALS AND METHODS**

**Metal salts.** Metal salts were obtained either as chloride or as nitrate salts from Fluka. Cytochrome c (horse heart) was from Sigma, prepared without trichloroacetic acid.

Bovine heart mitochondria were prepared according to Smith (7). bc1 complex was prepared as described by Schägger et al. (8) with the following modification: the buffer for the mitochondrial suspension (10 μM DTT, 20 μM NADH, 6 M urea, 100 mM sucrose, 50 mM Pipes, pH = 7.2) was added to the mitochondria and the bc1 complex was prepared following the procedure given by Schägger et al. (9). After complete elution of the iron-sulfur protein, Fe-S-deficient bc1 complex + antimycin was prepared by washing the mitochondria with 0.5% Triton X-100, 20 mM Pipes, pH 7.2, and run with the same buffer. The fractions containing Fe-S-deficient bc1 complex + antimycin were mixed with 10% (w/v) glycerol and frozen in liquid nitrogen.

For kinetic measurements was prepared as follows: 200 mM sucrose, 50 mM Pipes (from a partially neutralized stock solution), and 1 μM NaNO3 were dissolved in distilled water. The solution was purified over a Chelex 100 ion exchange resin column (analytical grade, Bio-Rad). 200 mM HNO3, 1 mM Ca(NO3)2, and 0.024% (0.5 mM) dodecyl maltoside (Boehringer) were added, and the pH was adjusted with 2M N-morpholino)propanesulfonic acid; Fe-S, iron-sulfur cluster; NBH, nonylubihydroquinone; Qp center, ubiquihydroquinone oxidation center at the positive P-side of the membrane; Qc center, ubiquinone reduction center at the negative N-side of the membrane; MOA, E-β-methoxyacrylate.

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1 The abbreviations used are: Pipes, piperazine-N,N′-bis(2-ethanesulfonic acid) Epsps, N-2-hydroxyethylpiperazine-N′-3-propanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; Fe-S, iron-sulfur cluster; NBH, nonylubihydroquinone; Qp center, ubiquihydroquinone oxidation center at the positive P-side of the membrane; Qc center, ubiquinone reduction center at the negative N-side of the membrane; MOA, E-β-methoxyacrylate.
Inhibition of isolated bc1 complex by various metal ions
Cytochrome c reduction by NBH was measured at different metal ion concentrations. K1 values were obtained by nonlinear curve-fitting, pH 7.0.

| Metal ion | K1 (μM) |
|-----------|---------|
| Zn2+ | 0.1 | Reversible |
| Ag+ | 1 | Reversible |
| Hg2+ | >1 | Not completely reversible |
| Cu2+ | >2 | Not completely reversible |
| Dy3+ | 4 | Reversible |
| Cd2+ | 7 | Reversible |
| Tb3+ | 8 | Reversible |
| Eu3+ | 12 | Reversible |
| Fe3+ | 20 | Reversible |
| Pb2+ | 30 | Reversible |
| Al3+ | >100 | Reversible |
| Ni2+ | >100 | Reversible |
| Co2+ | >100 | Reversible |
| Mg2+ | >1000 | No inhibition observed |
| Ca2+ | >1000 | No inhibition observed |
| Mn2+ | >1000 | No inhibition observed |
| Cr3+ | >1000 | No inhibition observed |
| Ti3+ | >1000 | No inhibition observed |
| Fe2+ | Direct reaction with cytochrome c |
| VO2+ | Direct reaction with cytochrome c |

ml of Chelex 100 purified buffer containing 50 mM Pipes, 20 mM HNO3, and 1 mM Ca(NO3)2, bc1 complex was added to a final concentration of 0.4 μM cytochrome b (approximately 70 μM). The bc1 complex was sedimented by centrifugation (Rotor Ti 70.1, 35,000 rpm, 3 h). 2 ml of the supernatant were withdrawn to measure free 65Zn. The remaining supernatant was discarded, and the tubes were centrifuged again (20,000 rpm, 30 min). The supernatant was removed and the pellet was incubated overnight in 50 μl of 10% Triton X-100, 1 mM NaCl. The dissolved pellet was diluted with 625 μl of buffer containing 0.5% Triton X-100, 200 mM NaCl, 20 mM Mops and finally with another 625 μl of water. Thus, the final volume was exactly one-half of the original assay solution. 1 ml (water added to a final volume of 2 ml) was used to measure bound 65Zn in a Beckman Gamma 5500 counter.

bc1 complex concentration was determined from reduced-oxidized difference spectra using ε562-575 = 28.5 μM cm⁻¹. EPR spectroscopy was done with a Bruker 200 D spectrometer equipped with cryogenics, peripheral equipment, and data acquisition/manipulation facilities as described previously (10).

RESULTS

Metal Ion Inhibition of Bovine Heart Mitochondria—Inhibition of succinate:O2 respiration was measured in uncoupled mitochondria washed with Chelex-purified buffer. Complex inhibition curves were obtained, indicating multiple metal binding sites. About 40% of the succinate:O2 activity was inhibited at low zinc concentration (<2 μM), but a concentration of >400 μM (500 Zn²⁻/bc1) was required for 90% inhibition. Hg²⁺, Ag⁺, Cu²⁺, and Cd²⁺ were found to be less effective in this order.

Inhibition of Isolated bc1 Complex—In contrast to whole mitochondria, simple inhibition curves were obtained when isolated bc1 complex was titrated with various metal ions. The strongest inhibition was observed by Zn²⁺ ions, followed by Ag⁺, Hg²⁺, and Cu²⁺ (Table I). The Ki for Cd²⁺ was 100 times higher than the Ki for Zn²⁺. Inhibition by Hg²⁺ and Cd²⁺ was not completely reversed by addition of the metal chelator, diethylthreitol/magnesium/cysteine, while inhibition by Zn²⁺ and Cd²⁺ was completely reversible.

Fig. 1 shows inhibition of NBH:cytochrome c oxidoreductase activity of isolated bc1 complex by Zn²⁺ ions. The Ki could be obtained by fitting the Zn²⁺ dependence with a single homogeneous inhibition site:

\[ v = V_{\text{max}} / (1 + [Zn^{2+}] K_i) \]  
(Eq. 1)

At pH 7.0, a K1 of 1 × 10⁻⁷ was obtained. A single inhibition site is also observed by plotting the linearized form

\[ 1/v = 1/V_{\text{max}} + [Zn^{2+}] / (K_i \cdot V_{\text{max}}) \]  
(Eq. 2)

At [Zn²⁺] > 5 μM, no steady state electron transfer activity was detectable.

Zn²⁺ Binding Assay Using ⁶⁵Zn—In the binding assay, multiple binding sites with different stoichiometry and affinity were observed (Fig. 2). The binding curve could be fitted by the standard binding equation assuming two independent types of binding sites (pH 7.2). High affinity site: N, 1.1 ± 0.1 Zn²⁺/c;i; K1, 0.13 μM; low affinity sites: N, 3–4 Zn²⁺/c;i; K1, 2.3 μM.

The K1 of the high affinity binding site was essentially identical with the inhibition constant Ki under all conditions tested. Therefore, we conclude that inhibition is caused by binding of a single zinc ion per cytochrome C1 with a dissociation constant of approximately 10⁻⁷ M. The additional multiple low affinity binding sites are not related to the inhibition of catalytic activity.

Note that the high affinity binding constant cannot be obtained by using a linear fit of the Scatchard plot (see Fig. 2, inset). A line representing only high affinity binding does not match the data points even at low zinc concentrations since approximately 15% of the first zinc ions bind to low affinity sites.

Inhibition of Single Turnover Cytochrome Reduction—No zinc inhibition of cytochrome b reduction through the QN center was observed in the presence of QP, inhibitors (not shown). Antimycin blocks electron transfer through the QN center so that only the QP center is active (11). In the presence of antimycin and 100 μM Zn²⁺, both cytochrome C1 reduction and cytochrome b reduction were significantly slower compared to the antimycin control (Fig. 3). The potent QP center inhibitor, MOA-stilbene, blocked both cytochrome C1 and cytochrome b reduction completely (12). The data show that zinc affects the QP but not the QN center.

Kinetics of Partially Inhibited bc1 Complex—In order to determine the inhibition mechanism, we tested whether zinc interferes with binding of either substrate, ubihydroquinone or cytochrome c. Michaelis-Menten enzyme kinetics of bc1 complex for NBH and cytochrome c were measured at zinc concentrations of 0, 0.075, and 0.2 μM corresponding to 0, 35, and 55% inhibition, respectively (Fig. 4). With both hydroquinone and cytochrome c, Vmax decreased while the Km values were unchanged as indicated by the parallel lines in the Eadie-Hofstee plot (Fig. 4). This shows that zinc does not interfere with the interaction of substrate and enzyme; inhibition occurs by a mechanism which is noncompetitive for both substrates.
Interaction between Zinc and Organic QP Center Inhibitors—

The interaction between zinc and the QP inhibitor, MOA-stilbene, was tested in two different ways. The binding constant for MOA-stilbene measured by fluorescence quench titration (13) was identical in the absence and presence of 100 μM Zn2+. The Kd, for zinc binding was identical for bc1 complex with or without bound MOA-stilbene (data not shown).

Binding of the QP center inhibitor, stigmatellin, which interacts with both the iron-sulfur protein and cytochrome b (14), was determined by red shift titration of reduced bc1 complex (15). The Kd could be determined exactly due to the high affinity; for bc1 complex both in the absence and presence of 100 μM Zn2+. Kd values below 20 nm were obtained. We conclude that zinc does not affect the binding of other QP center inhibitors.

pH Dependence of Zinc Binding and Inhibition—The pH dependence of zinc inhibition was tested by determining both the inhibition constant Ki and the high affinity dissociation constant Kd at different pH values. Both Kd (●) and Kd (▲) are less than 0.1 μM at pH > 7, while both values increased (i.e. affinity decreased) at lower pH values (Fig. 5). Since the values obtained by both methods were identical, we will use only the term Kd during the following discussion for values obtained by both inhibition and binding experiments.

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Inhibition of bc1 Complex by Zinc Ions

**DISCUSSION**

**Effect of Different Metal Ions on the bc1 Complex**—Of the metal ions tested, only Zn2+ and Ag+ bind with higher affinity to the bc1 complex than to other sites in isolated mitochondria. Binding of Zn2+ to the bc1 complex is more than 4 orders of magnitude tighter than binding of Co2+. This prevents spectroscopic investigation of the binding site as zinc ions are not amenable to most spectroscopic or magnetic techniques and is surprising since Co2+ (ionic radius 0.72 Å) generally is an excellent probe for natural zinc sites (ionic radius of zinc: 0.74 Å). The highest differences between the stability of zinc and cobalt complexes are observed for tetrahedral sites with nitrogen or sulfur ligands (19).

Binding of zinc is completely reversible. Studies using o-phenanthroline showed that reactivation requires 3 μmol of o-phenanthroline/μmol of Zn2+ added. Therefore, reactivation involves removal of zinc from the bc1 complex and complexation as Zn(phen)2+. Binding of Hg2+ and Cu2+ was not fully reversible, probably due to the oxidation of disulfide groups by both ions.

In isolated bovine mitochondria, the zinc content has been determined and with reduced cytochrome c added (data not shown). This shows that zinc binding is redox independent. Therefore, the group where protons bind with negative cooperativity to zinc must have a redox independent pKa value of 7.2.

**Does Zinc Bind to the “Rieske” Iron-Sulfur Protein?**—In order to test whether zinc binds to the iron-sulfur protein as reported previously (16), we measured zinc binding to Fe-S-depleted bc1 complex. This preparation is inactive due to loss of the iron-sulfur protein and has also lost the smallest subunit (6.4 kDa). Fe-S-depleted bc1 complex can be reactivated by addition of isolated iron-sulfur protein (17, 18) and still binds Qp center inhibitors (15).

Since Fe-S-depleted bc1 complex is prepared in the presence of antimycin (9), we first tested whether antimycin affects zinc binding. The KD observed with and without saturating concentrations of antimycin was identical (Fig. 7). The KD observed for the Fe-S-depleted bc1 complex was 3 times lower than for whole bc1 complex, i.e., binding is three times tighter (Fig. 7). The KD showed the same pH dependence as whole bc1 complex (data not shown). We conclude that zinc does not bind to the iron-sulfur protein but to residues in the vicinity of the iron-sulfur protein.

**Effect of Zinc Binding on the EPR Spectrum of the Rieske Iron-Sulfur Protein—**EPR spectra of bc1 complex in Chelex 100-purified nitrate buffer and reduced with dithionite were recorded with and without addition of saturating zinc concentrations ([bc1] = 76 μM; [Zn2+] = 173 μM; T = 17 K). The spectra without and with zinc added were indistinguishable (not shown).

**Redox Dependence of Zinc Binding**—Since the bc1 complex has several groups with redox dependent pK values, we tested the effect of the redox state of the “high potential chain” of the bc1 complex (iron-sulfur protein and cytochrome c) on zinc binding. The redox state was maintained during the 65Zn binding assay by addition of a 250-fold excess of oxidized or reduced cytochrome c (50 μM). At the end of the experiment, cytochrome c was still >90% in its original redox state. Electrons equilibrate rapidly between cytochrome c, cytochrome bc1, and the iron-sulfur protein. The redox state of cytochrome bc1 could not be determined in the presence of the high excess of cytochrome c.

The same dissociation constant KD for zinc was obtained in all three experiments: without cytochrome c added, with oxidized and reduced cytochrome c added (not shown). This shows that zinc binding is redox independent. Therefore, the group where protons bind with negative cooperativity to zinc must have a redox independent pKa value of 7.2.

**Fig. 5.** pH dependence of inhibition of bc1 complex by zinc ions. A: , Kc values obtained from inhibition kinetics; , KD values obtained from binding assays. The bold lines was fitted to the data points using the competitive binding model (see text) giving KD1 (high pH) = 0.07 μM; pKα = 7.2. The dotted and the dashed lines represent calculated curves for the noncompetitive binding model using KD2 = 0.07 μM; pKα = 7.2; KD/kD1 = 100 and 1000, respectively. B: same data as before. The curves were calculated for fully competitive inhibition using three different pKα values of 6.6, 7.2, and 7.6 (from left to right).

**Fig. 6.** pH dependence of electron transfer activity of bc1 complex. The line was fitted giving pKα = 6.6. Above pH 8.5, activity decreases.

Since it was not possible to measure either zinc binding or zinc inhibition at pH < 5.5, we cannot distinguish between competitive and noncompetitive binding models, if KD2/kD1 > 1000. However, in both cases, there is strong negative cooperativity between zinc and proton binding; on protonation, the dissociation constant increases by more than 2 orders of magnitude. This prevents spectroscopic investigation of the binding site as zinc ions are not amenable to most spectroscopic or magnetic techniques and is surprising since Co2+ (ionic radius 0.72 Å) generally is an excellent probe for natural zinc sites (ionic radius of zinc: 0.74 Å). The highest differences between the stability of zinc and cobalt complexes are observed for tetrahedral sites with nitrogen or sulfur ligands (19).

**Fig. 7.** Effect of different metal ions on the bc1 complex. The complexes were calculated for fully competitive inhibition using three different pKα values of 6.6, 7.2, and 7.6 (from left to right).
and are both involved in the formation of the Q.P action center. In addition, 3–4 low affinity sites per Mn with the experimental conditions used in the work of Lorusso et al. (16) that are likely to cause artifacts. The iron-sulfur protein, 42% decrease of the Rieske EPR signal irreversible inhibition of the QP center, binding of zinc to the zinc binding site resides on cytochrome bc1 where zinc inhibition occurs (15). Therefore, it is likely that the half-inhibition observed by Lorusso et al. (16) corresponds to a turnover number of 5–8 s⁻¹ for durohydroquinone as substrate using the specific bc1 content reported in Ref. 21. 50% inhibited activity corresponds to a turnover number of 2–4 s⁻¹. We have obtained turnover numbers in excess of 350 s⁻¹ using nonylubhydroquinone as substrate.

Zinc does not bind to the iron-sulfur protein since Fe-S-depleted bc1 complex, which can be reconstituted with iron-sulfur protein and does still bind MOA inhibitors to the QP center, binds zinc with 3 times higher affinity than bc1 complex. In addition, we have not observed any effect of saturating zinc concentrations on the EPR of the iron-sulfur cluster.

Zinc Does Not Compete with Either Cytochrome, Quinone, or Inhibitors, but Shows Negative Cooperation with Proton Binding—Despite showing noncompetitive inhibition kinetics, E-β-methoxyacrylates, known QP center inhibitors, increase the Km for durohydroquinone (12). In contrast, zinc does not affect binding of any of the substrates, cytochrome c or quinone; this is emphasized by the fact that zinc also does not compete with organic QP center inhibitors for a common binding site. This suggests that zinc has a mechanism of inhibition completely different from any other known bc1 inhibitor.

bc1 complex will bind protons and zinc with approximately equal affinity (Km(Zn²⁺)/Km(H⁺) ~ 1). Due to the strong negative cooperativity, zinc and protons displace each other from their binding sites. On zinc binding, the pKₐ of the proton binding site drops from 7.2 to below 5. However, this proton binding site is not related to the group determining the pH dependence of the activity of the bc1 complex, which depends on deprotonation of a group with pKₐ = 6.6 (Fig. 6) (21, 22). The pKₐ of the zinc binding site is also not identical with the redox dependent pKₐ of the oxidized Rieske iron-sulfur protein (pKₐ = 7.7) (23–26). Therefore, we conclude that we have identified another functionally important protonatable group of the bc1 complex.

Mechanism of Zinc Inhibition—Binding of zinc does not interfere with the binding of either of the substrates, hydroquinone or cytochrome c, but interferes with the binding of protons. Therefore, the most likely mechanism for inhibition by zinc is interference of zinc with proton transfer reactions at the QP center of the bc1 complex.

Hydroquinone oxidation requires the release of two protons from the QP center to the aqueous environment. The quinone reaction pocket itself must be shielded from the environment in order to prevent side reactions of the reaction intermediates with water or oxygen. Therefore, proton release will require a proton-conducting pathway to the aqueous phase (see Ref. 6). The pKₐ = 7.2 of the newly identified protonatable group is optimally suited for proton conduction at neutral pH. Since binding of zinc shifts the pKₐ value of the proton binding site from 7.2 to below 5, channelling of protons is inhibited by binding of zinc. Electron transfer is then inhibited by preventing release of the protons liberated in the redox reaction.

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Inhibition of bc₁ Complex by Zinc Ions

APPENDIX

Derivation of the Binding Equation for Zinc and Protons

The following scheme describes simultaneous binding of protons and zinc to enzyme E:

\[
\begin{align*}
\text{Zn} \quad & \quad \text{E} \quad \xrightarrow{\text{(1)}} \quad \text{E} \text{Zn} \\
\text{H}^+ \quad & \quad \text{EH} \quad \xrightarrow{\text{(3)}} \quad \text{EHZn} \\
\text{Zn} \quad & \quad \text{E} \quad \xrightarrow{\text{(4)}} \quad \text{E} \text{Zn} \\
\text{H}^+ \quad & \quad \text{EH} \quad \xrightarrow{\text{(2)}} \quad \text{EHZn}
\end{align*}
\]

**Scheme 1.**

The four dissociation constants are:

\[
K_{D1} = \frac{[E] \cdot [Zn]}{[EZn]} \quad \text{(high pH)}
\]

\[
K_{D2} = \frac{[EH] \cdot [Zn]}{[EHZn]} \quad \text{(low pH)}
\]

\[
K_a = \frac{[E] \cdot [H^+]}{[EH]}
\]

\[
K_p = \frac{[EZn] \cdot [H^+]}{[EHZn]}
\]

The total enzyme concentration, \(E_0\), is given by

\[
E_0 = [E] + [EH] + [EZn] + [EHZn]
\]

\[
[EHZn] \quad \text{can be expressed as}
\]

\[
[EHZn] = \frac{[Zn] \cdot E_0}{K_{D2} \left(1 + \frac{K_a}{[H^+]}\right) + [Zn] \left(1 + \frac{K_p}{[H^+]}\right)}
\]

Equation A4 gives \([EZn]\) as

\[
[EZn] = \frac{K_p}{[H^+]} \cdot [EHZn]
\]

The total fraction of enzyme with bound zinc, \((|EZn| + |EHZn|)/E_0\), is given by

\[
\frac{|EZn| + |EHZn|}{E_0} = \left(1 + \frac{K_p}{[H^+]}\right) \cdot \frac{|EHZn|}{E_0}
\]

\[
\frac{|EZn| + |EHZn|}{E_0} = \frac{[Zn]}{K_{D2} \cdot \frac{1 + \frac{K_a}{[H^+]} + [Zn] \left(1 + \frac{K_p}{[H^+]}\right)}{1 + [H^+]}}
\]

By comparing the binding equation without protons,

\[
\frac{|EZn|}{E_0} = \frac{|Zn|}{K_{D1} + |Zn|}
\]

we obtain the binding equation for \((|EZn| + |EHZn|)/E_0\) with protons by replacing \(K_{D1}\) with an apparent \(K_{D}'\) as follows:

\[
K_{D}' = K_{D2} \cdot \frac{1 + \frac{K_a}{[H^+]} + [Zn] \left(1 + \frac{K_p}{[H^+]}\right)}{1 + [H^+]}
\]

For \(K_{D2} \to \infty\), binding of zinc becomes competitive with proton binding. In this case, Equation A12 reduces to

\[
K_{D}' = K_{D} \cdot \frac{1 + \frac{[H^+]}{K_a}}{K_a}
\]

The same result is obtained if the \(K_{D}'\) for competitive binding is calculated directly.

REFERENCES

1. von Jagow, G. & Link, T. A. (1986) Methods Enzymol. 126, 224–237
2. Skałachev, V. P., Chistyakov, V. V., Jasaitis, A. A. & Smirnova, E. G. (1967) Biochim. Biophys. Acta 165, 109–126
3. Nicholls, P. & Malviya, A. N. (1968) Biochem. Biophys. Res. Commun. 26, 1–6
4. Kleiner, D. & von Jagow, G. (1972) FEBS Lett. 20, 229–232
5. Kleiner, D. (1974) Arch. Biochem. Biophys. 165, 121–125
6. Brandt, U. & Trumpower, B. L. (1994) CRC Crit. Rev. Biochem. Mol. Biol. 29, 165–197
7. Smith, A. L. (1967) Methods Enzymol. X, 81–86
8. Schägger, H., Link, T. A., Engd, W. D. & von Jagow, G. (1984) Methods Enzymol. 126, 224–237
9. Schägger, H., Borchard, U., Machleidt, W., Link, T. A. & von Jagow, G. (1987) FEBS Lett. 219, 161–168
10. Pierik, A. J. & Hagen, W. R. (1991) Eur. J. Biochem. 195, 505–516
11. von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T. & Trumpower, B. L. (1980) J. Biol. Chem. 255, 126, 6318–6326
12. Brandt, U., Schägger, H. & von Jagow, G. (1988) Eur. J. Biochem. 173, 499–506
13. Brandt, U. & von Jagow, G. (1991) Eur. J. Biochem. 195, 163–170
14. von Jagow, G. & Ohnishi, T. (1985) FEBS Lett. 185, 311–315
15. Brandt, U., Haase, U., Schägger, H. & von Jagow, G. (1991) J. Biol. Chem. 266, 19958–19964
16. Lorussi, M., Cocco, T., Sardanelli, A. M., Minuto, M., Bonomi, F. & Papa, S. (1991) Eur. J. Biochem. 197, 555–561
17. Trumpower, B. L., Edwards, C. A. & Ohnishi, T. (1980) J. Biol. Chem. 255, 1185, 7487–7493
18. Engel, W. D., Michalski, C. & von Jagow, G. (1983) Eur. J. Biochem. 132, 395–402
19. Mariet, W. & Vallee, B. L. (1993) Methods Enzymol. 226, 52–71
20. Wester, P. O. (1965) Biochim. Biophys. Acta 109, 263–283
21. Rieske, J. S. (1967) Methods Enzymol. X, 239–245
22. Rich, P. R. (1982) Faraday Discuss. Chem. Soc. 74, 349–364
23. Rich, P. R. (1984) Biochim. Biophys. Acta 768, 53–78
24. Prince, R. C. & Dutton, P. L. (1976) FEBS Lett. 65, 117–119
25. Link, T. A., Hagen, W. R., Pierik, A. J., Assmann, C. & von Jagow, G. (1992) Eur. J. Biochem. 208, 685–691
26. Link, T. A. (1994) Biochim. Biophys. Acta 1185, 81–84
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