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Effect of pH on CO recombination to cytochrome oxidase in intact mitochondria

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The rate of recombination of CO with fully reduced cytochrome oxidase in intact beef heart mitochondria was measured after flash photolysis at temperatures between 180 and 230K. At pH 7.4 a single Arrhenius slope corresponds to an apparent energy of activation (E_a) of 10 5 kcal/mol; the rate constants in 100% CO are twice those in the presence of 1% CO. At pH 5 5 with 100% CO, E_a's of 11 3 and 7 1 kcal/mol are observed above and below 210K, respectively, while E_a's of 7.4 and 11.1 kcal/mol are observed with 1% CO above and below 210K. At pH 9 0 E_a's of 9.2 (above 200K), 12.5 (190–200K), and 2.3 (below 190K) kcal/mol are observed with 1% CO; E_a's of 9.4, 13 4, and 2.4 kcal/mol are observed in the same temperature ranges with 100% CO present. The findings support a model with up to 4 energy barriers separating the heme region from the bulk medium with intermediate regions that can hold 1 or 2 CO, depending on pH.

Cytochrome oxidase, Copper; CO recombination, pH; proton

1. INTRODUCTION

Cytochrome oxidase (E.C. 1.9.3.1), a multi-subunit enzyme containing two hemes and two copper atoms, is responsible for the reduction of molecular oxygen to water in the mitochondrial respiratory chain. The reduction of oxygen requires three substrates to be present at cytochrome a_3: molecular oxygen, electrons donated from the respiratory chain via cytochrome *c*, and protons. Protons play three distinct roles in the operation of the oxidase: (i) substrate protons required in the reduction of oxygen; (ii) protons chemiosmotically translocated across the membrane in energy coupling; and (iii) those protons involved in or that determine the three dimensional conformation of the enzyme. Clearly, changes in proton conformation with varying pH may affect the utilization of protons in respiration and chemiosmosis.

The recent report by Fabian and Malmstrom [1] of the pH-induced spectral changes in oxidized oxidase lead those investigators to suggest that proton binds at or near cytochrome a_3. Proton binding near cytochrome a_3 may alter the kinetics of ligand binding as well. Data contained in the report of Brzezinski and Malmstrom [2] and earlier work from this group [3] suggested that CO recombination was not affected by changes in pH although a dedicated in-depth study was not performed. In this communication we report the pH-dependence of the energy of activation and number of CO molecules involved in CO recombination in cytochrome oxidase in intact beef heart mitochondria.

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2. MATERIALS AND METHODS

Intact beef heart mitochondria prepared by the procedure of Harmon and Crane [4] were suspended at 5 mg protein/ml in 0.25 M sucrose–50 mM sodium phosphate buffer at the desired pH (5.5, 7.4, or 9.0). The mitochondrial suspension was reduced by the addition of 28 μM tetramethyl-*p*-phenylenediamine dihydrochloride, 97 μM phenazine methosulfate, 50 mM succinate, 100 mM ascorbate (final concentrations), and 200 μg cytochrome *c* (Sigma type VI) while bubbling the solution with either 1% (99% N_2) or 100% CO. The suspension was bubbled with CO for 20 min in the dark and transferred to the sample holder; the loaded sample was allowed to sit in the dark for an additional 15 min and then frozen in liquid nitrogen.

CO recombination following flash photolysis was measured at 446 nm as described previously [5–7]. The resulting time-dependent absorbance changes were fitted to a single exponential curve using the Kinetics module of the Labcalc (TM) program (Galactic Industries) and the program KINFIT (On-Line Instrument Systems, Inc., Jefferson, GA). That lower standard deviations and higher Durbin–Watson values are obtained from single exponential than multiple exponential or power-law fits indicates that the re-binding can be described by a single exponential as before [5–7].

That the oxidase was totally reduced and ligated to CO was determined from reduced minus oxidized difference spectra measured with a Johnson Research Foundation (University of Pennsylvania) model DBS-3 scanning dual wavelength spectrophotometer

3. RESULTS

Plots of the log of the rate constant *k* vs inverse temperature at pH 7.4, 5.5, and 9.0 are shown in Figs.

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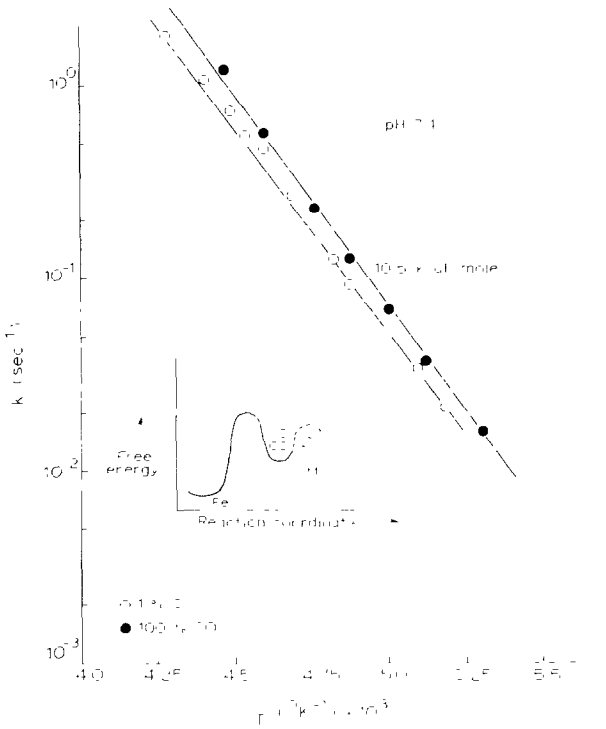

Fig. 1 Plot of log k of CO recombination vs inverse temperature at pH 7.4 in intact beef heart mitochondria. The calculated energies of activation are shown adjacent to the slopes. The fitted lines (least square fit) indicate energies of activation of 10.5 kcal/mol (coefficients of correlation for both lines are greater than 0.994). The standard error of measurements of k, unless shown as an error bar, lies within the data point dot shown on this and succeeding graphs.

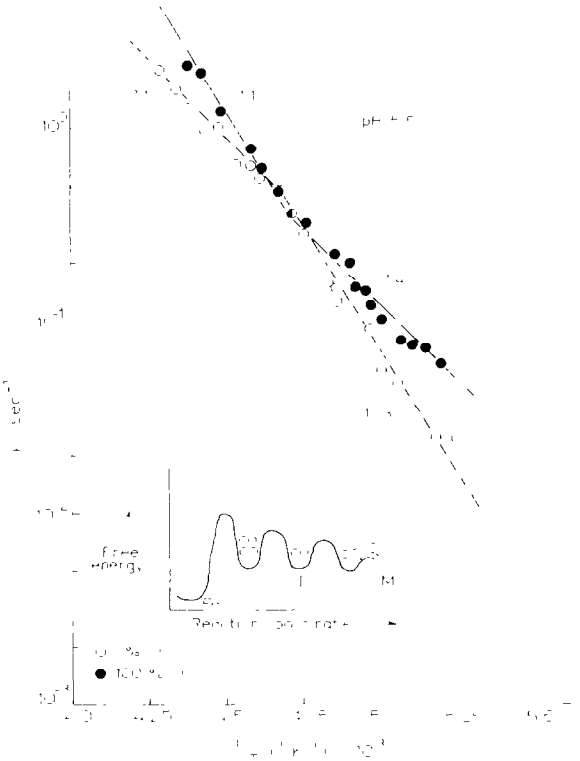

Fig. 2. Plot of log k vs inverse temperature at pH 5.5. The calculated energies of activation are shown adjacent to the slopes. The coefficients of correlation of fit of the drawnlines with 1% CO above and below 215K are both 0.99; the coefficients with 100% CO above and below 215K are 0.998 and 0.985, respectively.

1–3. The apparent energies of activation are indicated adjacent to the least-squares fitted lines on the plots (coefficients of correlation are given in the legends). In the presence of 1% and 100% CO, a monophasic 'Arrhenius plot' is observed at pH 7.4. The values of k approximately double in the presence of 100% CO (compared to 1% CO). Similar plots are obtained at pH values between 6 and 8.5 (data not shown). At pH 5.5, apparent biphasic plots are observed in the presence of 1% and 100% CO.

The plots obtained at pH 9 are strikingly different. In the presence of 1% CO a triphasic plot is observed while in the presence of 100% CO, a 4-phase plot is observed. The values of k with 100% CO are higher than with 1% CO at temperatures above 210K, the difference increasing with increasing temperature.

If the pH of the intact mitochondrial suspension is readjusted from pH 5.5 or 9.0 back to pH 7.4, a monophasic Arrhenius plot with 10.6 kcal/mol energy of activation is observed. In addition, the values of k calculated in the reneutralized samples are coincident with those observed in samples originally suspended at pH 7.4 (cf. Fig. 1). This indicates that the pH-induced effects are reversible and that non-reversible denaturation or conformational changes are not involved.

4. DISCUSSION

The Arrhenius plots of the rate constants measured in the presence of 1% and 100% CO convey different pieces of information concerning CO recombination. In the presence of 1% CO, only one CO is present per oxidase on average; after photolysis, recombination of the single (previously bound) CO with the heme iron requires crossing thermodynamic barriers separating intermediate CO-holding (not binding) regions. The rate of CO movement from one region to another is a function of the concentration of CO present as well as the height of the energy barriers encountered. Increasing the number of CO molecules in a region will increase the concentration of CO and the rate of CO migration from that region assuming the barrier heights are not altered by the increased CO concentration. The concentration dependence of the value of k indicates the number of CO molecules present in each intermediate region (the occupancy). If a region can hold 2 CO, then the rate of CO migration from that region will be proportionally faster. The binding of CO to the oxidase is not truly second order, since a 100-fold increase in CO concentration results in only a two-fold increase in binding rate. Thus the Arrhenius plot in the presence of

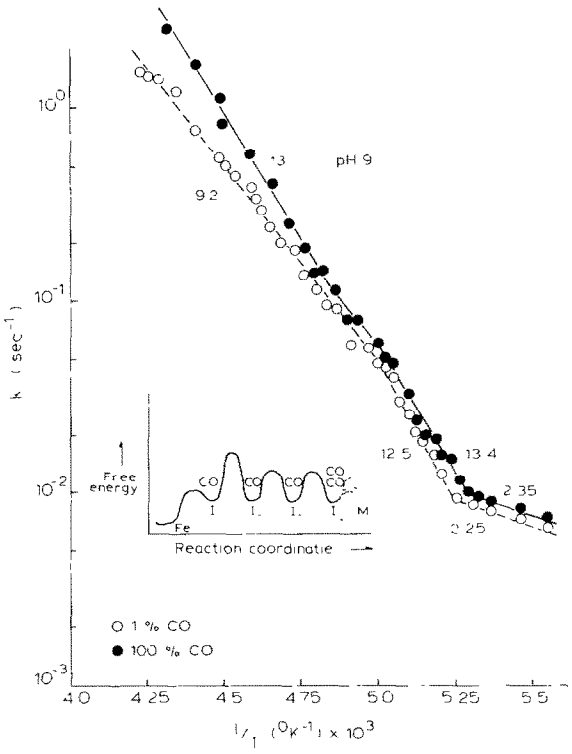

Fig. 3. Plot of log k vs inverse temperature at pH 9.0. The energies of activation are shown. The coefficients of correlation of the fitted lines with 1% CO are, from warmer to colder temperatures, 0.998, 0.982, and 0.995. The coefficients with 100% CO from warmer to colder temperatures are 0.992, 0.991, 0.966, and 0.986, respectively.

1% CO indicates a minimum number of barriers and defines their heights while plots in the presence of 100% CO indicate the occupancy or number of CO molecules in the intermediate regions separated by the barriers. This interpretation of the data is consistent with previous interpretations by this and other investigators [7,8] with cytochrome oxidase.

We interpret the results by the reaction coordinate models (included as insets of Figs. 1–3) as in previous reports with cytochrome oxidase [7,8] and myoglobin [9,10]. CO migration to the heme at pH 7.4 entails crossing one or more barriers of 10.5 kcal/mol from one or more intermediate regions that can hold 2 CO maximally. Since the kinetics we would observe with one barrier/region are indistinguishable from those with multiple identical barriers/regions, we will assume 4 barriers and regions (other than the heme and bulk solvent denoted 'M', the barrier to which is temperature-dependent) to be consistent with the number of barriers observed at pH 9.

At pH 5.5, CO must cross at least 3 barriers. The innermost barrier (and the last to be frozen at low temperatures) is 11.3 kcal in height and separates the Fe from the innermost region I; region I can contain 2 CO. Between 205K and 210K, CO molecules migrate from region I_2 which has an occupancy of 1 CO since the values of k are the same in 1% and 100% CO; additional identical regions may be present. An outermost region I_3, which because of its larger k-value in 100% CO has an occupancy of 2, is separated from I_2 by a 7.1 kcal barrier. Migration to the heme from I_3 via the other intermediate regions occurs above 215K.

At pH 9, the presence of three slopes with 1% CO indicates a minimum of three energy barriers. The values of k are greater with 100% CO only above 210K. Thus three inner regions I, I_2, I_3 can each hold 1 CO and are separated by 2.3 (Fe-I), 12.5 (I-I_2), and 9.2 kcal/mol (I_2–I_3) barriers. Region I_3 is separated from the outermost region I_4 (occupancy = 2) by a 9.2 kcal/mol barrier. Regions I_3 and I_4 are distinguishable because of their difference in occupancy, not their difference in barrier heights.

The pH-dependent changes observed here are likely due to pH-dependent conformational changes and not due to translocated protons since proton translocation cannot occur without enzymic turn-over. Alternatively, the changes observed may be related to the binding of substrate protons at the cytochrome a_3–CuB binuclear complex in preparation for their transfer to the oxygen. The alterations in barrier size and region occupancy could be due to protonation/deprotonation of 1 or more amino acid residues in the pocket or channel to the protein exterior. Doster et al. [10] observed pH-dependent CO binding to myoglobin and the beta-chain of hemoglobin, attributed to a single titratable residue with a pK of 5.7, likely the distal histidine. That the plots between pH 6 and 8.5 are not different than that observed at pH 7.4 (in agreement with studies by Wohlrab and Ogunmola [11] suggests that the change at pH 9 may be due to deprotonation of a tyrosine or cysteine residue and the change observed at pH 5.5 may be due to deprotonation of a histidine residue. Fabian and Malmstrom [1] and Papadopoulos et al. [12] have suggested that changes in Soret absorbance maxima may be due to protons associating with the histidine of cytochrome a_3.

Oliveberg et al [13] reported that the effects of pH on intra-oxidase electron transfer in mixed valence isolated oxidase were not due to pH dependence on the formation of the 'oxy' form, although the authors concede a large experimental uncertainty in their measurement of rates from room temperature flow-flash experiments. The stimulation of both CO and oxygen binding to reduced pigeon heart mitochondrial oxidase following 'energization' by ATP hydrolysis suggests that oxygen binding closely parallels CO binding [7,14]; we thus expect that the formation of the oxygenated ferrous intermediate compound A [15] would also show pH-dependent rates of formation at low temperatures; those experiments are being performed.

The overall rates of ligand binding at room temperature are reported to be pH-insensitive [13];

room temperature rate measurements cannot discern the rate constants of CO migration from region to region, while measurements at decreasing temperatures allow us to investigate processes sequentially nearer the heme [8-10]. The pH-dependent differences we observe may reflect alterations in the mechanism of binding of CO, perhaps in the transfer of CO from CuB to cytochrome a_3 [16-18]. These changes may reflect conformational changes in the protein or the formation of a protonated intermediate, either of which may play a role in the reduction of oxygen and gating of electron flow [1,19] and less likely (due to the pH-insensitivity of electron transfer in mixed valence oxidase) play a role in proton translocation.

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REFERENCES

[1] Fabian, M. and Malmstrom, B.G. (1989) Biochim. Biophys. Acta 973, 414-419.
[2] Brzezinski, P. and Malmstrom, B.G. (1987) Biochim. Biophys. Acta 894, 29-38.
[3] Harmon, H.J. (1977) Fed. Proc. 36, 900a.
[4] Harmon, H J. and Crane, F.L. (1974) Biochem. Biophys. Res. Comm. 59, 326-333.
[5] Harmon, H.J. (1988) Bull. Environ. Contam. Toxicol. 40, 105-109.
[6] Harmon, H J. (1988) J. Bioenerg. Biomemb. 20, 735-748.
[7] Harmon, H.J. and Sharrock, M. (1978) Biochim. Biophys. Acta. 503, 56-66.
[8] Sharrock, M. and Yonetani, T. (1977) Biochim. Biophys. Acta 462, 718-730.
[9] Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H., and Gunsalus, I.C., (1975) Biochemistry 14, 5355-5373.
[10] Doster, W., Beece, D., Browne, S.F., Dilorio, D.E., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., Winterhalter, K.H. and Yue, K.T. (1982) Biochemistry 21, 4831-4839.
[11] Wohlrab, H. and Ogunmola, G.B. (1971) Biochemistry 10, 1103-1106.
[12] Papadopoulos, P., Walter, S., and Baker, G (1990) Biophys J. 57, 237a.
[13] Oliveberg, M., Brzezinski, P. and Malmstrom, B.G (1989) Biochim. Biophys. Acta 977, 322-328.
[14] Harmon, H.J. and Wikstrom, M.K.F. (1978) Biochim. Biophys. Acta 503, 67-77.
[15] Chance, B., Saronio, C. and Leigh, J.S., Jr. (1975) J. Biol. Chem. 250, 9226-9237.
[16] Alben, J.O., Moh, P.P., Fiamingo, F.G. and Altschuld, R.A (1981) Proc Natl. Acad. Sci. 78, 234-237.
[17] Fiamingo, F.G., Altschuld, R.A., Moh, P.P and Alben, J.O. (1982) J. Biol. Chem. 257, 1639-1650.
[18] Dyer, R.B., Einarsdottir, O., Killough, P.M., Lopez-Garriga, J.J. and Woodruff, W.H. (1989) J. Am. Chem. Soc. 111, 7657-7659.
[19] Chan, S.I. and Li, P M. (1990) Biochemistry 29, 1-12.