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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

IX. REACTION OF CYTOCHROME aa_3 WITH HYDRAZINE

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SUMMARY

1. Aerobic incubation of purified cytochrome aa_3 with hydrazine, even in less than stoichiometric amounts, produces one of the so called 'oxygenated' forms of the enzyme *via* an O_2^- intermediate.

2. The O_2^- ion is not a universal intermediate in the formation of 'oxygenated' cytochrome aa_3.

3. Oxidation of hydrazine by cytochrome aa_3 follows Michaelis-Menten kinetics with a K_m of 3.8 mM and a maximum turnover number of 0.07 electron per mole cytochrome aa_3 per s at pH 7.2 and 20 °C.

4. The reactions mentioned under 1 and 3 differ in kinetic pattern, K_m for oxygen and sensitivity towards superoxide dismutase.

5. Hydrazine is a competitive inhibitor of cytochrome *c* oxidation by cytochrome aa_3 with a K_i of 25 mM at pH 7.2 and 20 °C.

The interaction of cytochrome *c* oxidase (ferrocytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1) with the classical inhibitors cyanide, azide, sulphide and CO has been extensively studied. However, little attention has been paid to hydrazine, except by the group of Okunuki[1-3]. The hydrazine molecule has three properties that may be relevant to its reaction with cytochrome aa_3: it is a strong reducing agent, a potential heavy-metal chelator and an aldehyde reagent. The last-mentioned property was especially emphasized by Okunuki and co-workers in an attempt to explain the complicated spectral characteristics of cytochrome *c* oxidase in terms of one haem–iron component, a copper atom and a reactive formyl side chain of porphyrin *a*. This 'unitarian' concept is at variance with the widely accepted view that two haem components, *a* and a_3, are present.

The previous work on hydrazine indicated little or no spectral changes with the oxidized enzyme[1,2] and a competitive type of inhibition of the cytochrome *c* oxidation[1]. Recently Orii and Yoshikawa[3] found evidence for two cooperative hydrazine-binding sites.

Addition of 0.1 mM hydrazine to cytochrome aa_3 (purified from beef heart-muscle preparation according to refs 4 and 5) shifts the γ-band of the oxidized

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enzyme from 424 to 427 nm and increases the absorbance in the α-band region slightly. The difference spectrum (oxidized $aa_3 + N_2H_4$ *minus* oxidized aa_3) shows peaks at 434, 534, 578 and 605 nm and troughs at 411, 486 and 650 nm (Fig. 1). The spectrum resembles that for cyanide[6] and is identical to that of the 'oxygenated' form[7–13], another conformation of the oxidized enzyme (see refs 12 and 13), produced by aeration of a dithionite-reduced sample.

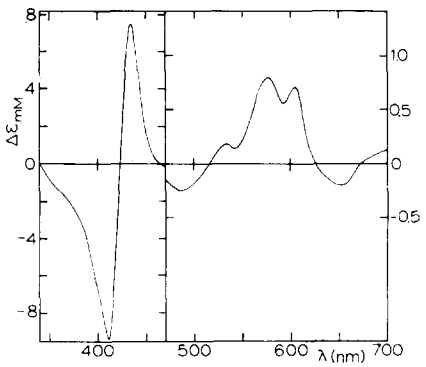


Fig. 1. Difference spectrum of oxidized cytochrome aa_3 + 100 μM hydrazine *minus* cytochrome aa_3 in 80 mM potassium phosphate (pH 7.2) and 0.7% cholate. Spectrum taken 25 min after hydrazine addition. Extinction coefficients are based on cytochrome aa_3 with two haem groups.

It is important to note that in the absence of oxygen hydrazine does not bring about specific spectral changes, but slowly reduces the enzyme when present in high concentrations. The reversibility of the aerobic reaction of cytochrome aa_3 with hydrazine was tested by evacuation to remove the oxygen or by gel filtration to remove the hydrazine. In both cases the γ-peak shifts back to 424 nm rather slowly, in a first-order reaction with the same rate constant ($5 \cdot 10^{-4}$ s^{-1} at 20 °C) as found for the 'oxygenated' form[14]. Moreover, like the 'oxygenated' form, the hydrazine-incubated enzyme is not reduced by NADH *plus* phenazine methosulphate in an anaerobic Thunberg cuvette. The overall oxidation state of the hydrazine-induced compound, determined with a titration method described earlier[14], is equal to that of the 'oxygenated' and oxidized enzyme.

Although the difference spectra for oxidized $aa_3 + N_2H_4$ *minus* oxidized aa_3 and 'oxygenated' aa_3 *minus* oxidized aa_3 are identical in shape, the absorbance coefficients in the former are about 35 % of those in the latter: 7 and 20 $mM^{-1} \cdot cm^{-1}$, respectively, at 432 nm. The same small changes are observed when H_2O_2 reacts with the oxidized enzyme ($\Delta A_{432\ nm} = 6\ mM^{-1} \cdot cm^{-1}$). This is in agreement with the conclusion of Orii and King[15] that several species of 'oxygenated' cytochrome aa_3 exist. The low absorbance coefficients in the difference spectrum with hydrazine are not due to incomplete conversion of the enzyme, caused by a steady-state level balancing the formation of the compound by hydrazine and its subsequent decomposition, since the final absorbance reached is independent of the hydrazine concentration between 10 μM and 10 mM N_2H_4. Thus, there is sufficient evidence to identify the product of hydrazine action in the presence of O_2 as an 'oxygenated' form, but the question remains whether this product simply results from oxidation of the hydrazine-reduced enzyme by oxygen. It is striking that reduction of aa_3

by hydrazine under anaerobic conditions followed by aeration produces a compound with an absorbance coefficient of 18.4 $mM^{-1} \cdot cm^{-1}$ at 432 nm, close to that found for the classical ($Na_2S_2O_4$/air) 'oxygenated' compound.

In Fig. 2 the reciprocal rate of oxygen consumption of cytochrome *c* oxidase in the presence of high concentrations of hydrazine is plotted against the inversed hydrazine concentration. From the intercepts of the resulting straight line a K_m of

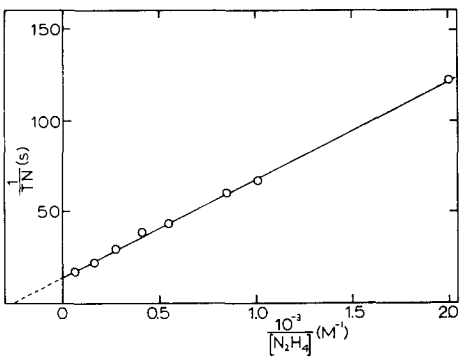


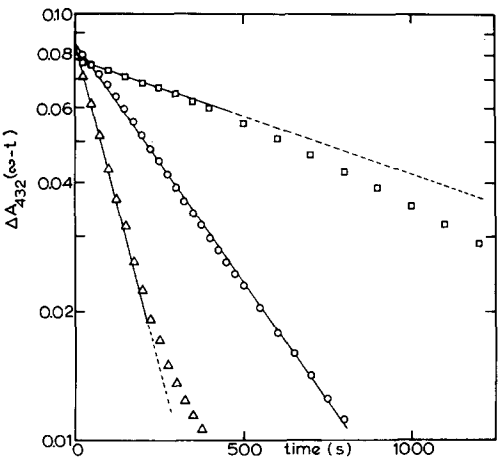
Fig. 2. Oxidation of hydrazine by cytochrome aa_3 (40 μM) in the presence of 3 μM superoxide dismutase. O_2 uptake was measured with a Clark electrode on a Gilson oxygraph. The reaction mixture contained 100 mM potassium phosphate (pH 7.2) an 0.5% Tween 80, temperature 20 °C. TN = turnover number.

3.8 mM and a turnover number at infinite hydrazine concentration of 0.072 electron per mole aa_3 per s is calculated. Oxidation of hydrazine by cytochrome aa_3 is fully sensitive towards azide and cyanide. Hydrazine is a poor substrate for cytochrome aa_3 when compared with cytochrome *c* (turnover number, 240 s^{-1})[16]. Hydrazine is a purely competitive inhibitor of cytochrome *c* oxidation (not shown) with a K_i of 25 mM at pH 7.2, 20 °C. Competitive inhibition was also observed by Takemori *et al.*[1] but our K_i value is about 50 times higher and is close to that calculated from the data of Orii and Yoshikawa[3].

In contrast to the normal zero-order Michaelis–Menten kinetics of hydrazine oxidation the spectral changes following addition of low concentrations of hydrazine to oxidized cytochrome aa_3 show complex kinetics. Since the initial velocity as monitored at 432 or 578 nm is proportional to the enzyme concentration (not shown) the reaction is first order in cytochrome aa_3. With hydrazine concentrations between 40 μM and 1 mM the reaction is first-order (Fig. 3, ○—○) but the observed first-order rate constant is proportional to the square root of the hydrazine concentration rather than to the concentration itself. The same conclusion is drawn from the observed slope ($n = 0.56$) of a line in a plot of the logarithm of the initial velocity against the logarithm of the hydrazine concentration (Fig. 4). The reaction order in hydrazine depends on the hydrazine concentration being nearly zero order at low concentrations ($\leqslant$ 10 μM) and approximately first order at concentrations above 1 mM.

Changes in the reaction order are also seen in Fig. 3. At high concentrations of hydrazine (1 mM) the line is convex to the time axis (△—△), suggesting that more than one step is involved in the reaction of hydrazine with cytochrome aa_3 (see

also ref. 6). At a concentration of hydrazine stoichiometric with cytochrome aa_3 (10 μM) the reaction becomes zero order as can be seen from the downward inflection of the line in Fig. 3 (□—□). This indicates that hydrazine plays a catalytic role in the formation of the 'oxygenated' compound. In line with this is the observation that with 0.4 mole hydrazine per mole cytochrome aa_3 the maximal absorbance change is reached in 1 h.



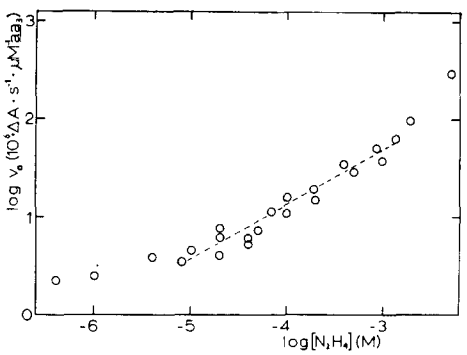


Fig. 3. Semilogarithmic plot of the time course of the reaction of hydrazine (added at $t = 0$) to cytochrome aa_3 (10.7 μM) in 80 mM potassium phosphate (pH 7.2) and 0.7% cholate. The ordinate gives the difference between the final $A_{432\ nm}$ and that at time t; temperature 20 °C. □—□, 10 μM; ○—○, 100 μM; and △—△, 1 mM hydrazine.

Fig. 4. Double logarithmic plot of the initial velocity of the reaction of cytochrome aa_3 with hydrazine as a function of the hydrazine concentration. 10.7 μM cytochrome aa_3 in 80 mM potassium phosphate (pH 7.2) and 0.7% cholate, temperature 20 °C.

Ferricytochrome *c*, known to accelerate the decomposition of 'oxygenated' cytochrome aa_3[11,17], completely prevents the spectral shifts of the oxidized enzyme with hydrazine, but does not react with hydrazine itself. The necessity of oxygen for the hydrazine reaction led us to investigate whether cytochrome *c* acts as scavenger of an O_2^- intermediate. This is proved by the strong inhibition of the spectroscopically visible reaction of cytochrome aa_3 with hydrazine by superoxide dismutase, purified from beef erythrocytes[18]. The reaction of 20 μM cytochrome aa_3 with 40 μM N_2H_4 is 90% inhibited by 1 μM superoxide dismutase. Complete inhibition is not to be expected since the H_2O_2 formed from O_2^- also causes 'oxygenation'[8,14,17,19], albeit less efficiently. In the EPR spectra of frozen mixtures of cytochrome aa_3 and hydrazine no O_2^- lines could be detected, indicating that no O_2^- is accumulated during the reaction.

Does the involvement of O_2^- mean that every O_2^--ion-producing system converts oxidized cytochrome aa_3 to the 'oxygenated' form? Indeed the well-known system[20] xanthine *plus* xanthine oxidase (purified from chicken liver[21]) forms 'oxygenated' cytochrome aa_3. However, superoxide dismutase is a poor inhibitor of this reaction whereas catalase (Sigma, from beef liver) is quite effective. Thus, 'oxygenation' of cytochrome aa_3 by xanthine *plus* xanthine oxidase is primarily

caused by H_2O_2 and not by O_2^-. Furthermore we are unable to demonstrate an effect of superoxide dismutase on the formation or decomposition of 'oxygenated' cytochrome aa_3 prepared by aerating a dithionite-reduced sample. It is, therefore, concluded that formation of 'oxygenated' cytochrome aa_3 does not necessarily proceed *via* the O_2^- ion although the latter is an intermediate in the case of hydrazine.

The spontaneous quantitative decomposition of 'oxygenated' cytochrome aa_3 to the normal oxidized form[11,14,17,22] indicates that the 'oxygenated' conformation is of higher energy content. Our experimental evidence for 'oxygenation' by catalytic amounts of hydrazine seems to violate this hypothesis. A way out may be provided by the observation of Hatefi and Hanstein[23] and confirmed by us[13] that the purified cytochrome aa_3 continuously consumes oxygen, possibly as a results of endogeneous phospholipid (per)oxidation. If the resulting electron flow proceeds via the haem groups and is catalysed by hydrazine the origin of the energy required for producing an 'oxygenated' conformation with slightly higher energy content might be explained. If this is true, oxygen would be necessary in stoichiometric amounts. Some evidence in favour of this is provided by the observation that half the normal absorbance change with 100 μM hydrazine and 10 μM cytochrome aa_3 was obtained when the oxygen concentration was lowered to about 4 μM.

In summary, two different effects of hydrazine on cytochrome aa_3 may be distinguished: (1) The formation of the 'oxygenated' enzyme *via* an O_2^- intermediate with even substoichiometric amounts of hydrazine, and sensitive to superoxide dismutase and cytochrome *c*. (2) The oxidation of hydrazine (presumably to N_2 and NH_3) by the enzyme, with a high K_m value (3.8 mM), fully inhibited by azide and cyanide but not by superoxide dismutase. Both reactions require oxygen. The K_m for oxygen for the oxidation of hydrazine is below the limits of our measurement as is the case with cytochrome *c* oxidation. For the 'oxygenation' reaction the K_m for oxygen is much higher. Both the initial velocity and the first-order rate constant are decreased when the oxygen concentration is lowered from 250 to about 4 μM. No indication was found that hydrazine remains bound either to the formyl group as hydrazone or as a ligand of the iron or copper atoms.

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