Reaction of oxygen with hydroxylamine oxidoreductase of *Nitrosomonas*

Fast kinetics

Alan B. Hooper and Claude Balny*

Department of Genetics and Cell Biology, University of Minnesota, St Paul, MN 55108, USA and **INSERM U128, Route de Mende, BP 5051, 34033 Montpellier Cedex, France*

Received 9 June 1982

Hydroxylamine oxidoreductase *Fast kinetics* Nitrosomonas *Oxygen*

1. INTRODUCTION

The chemolithotrophic bacterium *Nitrosomonas* oxidizes ammonia to nitrite with hydroxylamine as intermediate. The enzyme hydroxylamine oxidoreductase (HAO) catalyzes the 2 e^- dehydrogenation of NH_2OH (forming HNO) and subsequent net addition of an O atom from dioxygen to form HNO_2. The enzyme is an $\alpha_3\beta_3$ hexamer [1]: α contains 1 CO-binding heme P460 [2,3] and 6 *c*-type hemes; β contains 1 *c*-type heme. The substrate NH_2OH apparently binds at or near the heme P460 center and electrons are transferred to enzyme *c*-hemes from heme P460 [4].

Because ferrous P460 binds CO, one would expect that it also binds O_2 and may be involved in activation of dioxygen [5,6] for oxygenation of nitrogenous compounds such as HNO (and possibly NH_3). Activation of dioxygen may very likely involve initial 2 e^- reduction of O_2 prior to bond cleavage (see [17]). Ferrous hemes of HAO are rapidly oxidized by H_2O_2 (Hooper, A.B., Debey, P., Andersson, K.K. and Balny, C. unpublished). It has not been possible to study reaction of ferrous hemes of HAO with O_2 because full reduction of the enzyme usually required an excess of reductant (e.g., $Na_2S_2O_4$ or acridine orange) which, when mixed with O_2, produce H_2O_2. Thus, one could not confidently state that reoxidation was by reaction with O_2 as opposed to H_2O_2. This difficulty has been overcome in the present work by the use of photoreduced deazalumiflavin (DALF) as reductant. Because the reducing species of DALF disappears immediately upon cessation of illumination [8] it is possible to rule out the production of H_2O_2 after mixing of anaerobically reduced HAO and oxygenated solution.

We report the reaction of HAO (photoreduced with DALF) with oxygen at a rate commensurate with the turnover rate for HAO in vitro and in vivo, the partial identification of the product of O_2 reduction, the identification of site of O_2 reaction on HAO and the sequence of reoxidation of heme P460 and *c*-hemes with α absorption maxima at 552 and 558 nm.

2. MATERIALS AND METHODS

2.1. *Enzyme and chemicals*

Pure HAO was prepared from *Nitrosomonas* as in [2]. Pure cytochrome *c*-peroxidase was a gift of Dr T. Yonetani, University of Pennsylvania (prepared according to [9]). Deazalumiflavin was a gift of Dr J.M. Lhoste. Carbon monoxide, argon and nitrogen contained <3 ppm O_2. EDTA and bovine liver catalase (C 40) were from Sigma.

2.2. *Photoreduction of enzyme*

A volume of enzyme (~ 1.2 μM HAO; ~ 30 μM total heme), 0.1 M Na/K-phosphate solution (pH 7.5) 18 μM deazalumiflavin (from a 1 mM stock solution in dimethylformamide), 10 mM EDTA and 1.7×10^3 U/ml catalase was degassed by bubbling with Argon for 20 min. The solution was transferred into the drive syringe of the stopped-flow apparatus, degassed for an additional 30 min and illuminated for 30 min with a 120 W halogen lamp at 15 cm from the syringe. In a typical series of

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

stopped-flow experiments over 4 h, the photoreduction was supplemented with 4 periods of 10 min illumination.

2.3. *Kinetics of reoxidation of photoreduced enzyme*

Kinetic measurements were made with an anaerobic stopped flow mixing apparatus (designed by C. B.; built in Montpellier) adapted to the Aminco DW-2 spectrophotometer [10]. Kinetic constants have an estimated error of ±20%. The temperature of syringes and mixing chamber was maintained at + 20 ±0.1°C with a circulating bath from Colora Messtechnik.

Because the spectrophotometer was used in the dual-beam mode, 700 nm (where neither HAO or the CCP–H_2O_2 complex absorb) was chosen as reference wavelength. Wavelength combinations for the various pigments were as follows: reduced heme *c*-552, 552 minus 700 nm; reduced heme *c*-558, 560 minus 700 nm; reduced heme P460, 462.5 minus 483 nm. The latter wavelength pair was selected because the increase in absorbancy associated with oxidation of *c* hemes (concomitant with P460 oxidation) was about the same at 462.5 and 483 nm. For measurement of H_2O_2 production, reduction of HAO *c* hemes (assayed at 522 nm, an isobestic point for formation of CCP–H_2O_2 complex, minus 700 nm) was compared with formation of CCP–H_2O_2 complex (assayed at 436 nm, an isobestic point for HAO *c* heme reduction, minus 700 nm). Extinction coefficients used were: CCP–H_2O_2 minus CCP, ΔE_{438} = 17.5 $mM^{-1} \cdot cm^{-1}$; HAO, total *c* heme reduced minus oxidized, ΔE_{422} = 4.0 $mM^{-1} \cdot cm^{-1}$.

Rapid scan measurements were carried out with a Union Giken (Osaka), model RA 415 and RA 401 fast response spectrophotometer [11] equipped with the anaerobic stopped flow device (maximum recording speed: 95 nm/ms).

3. RESULTS AND DISCUSSION

3.1. *Kinetics of oxidation of HAO hemes*

Stopped-flow mixing of DALF-reduced HAO with air-saturated solution (final conc. 0.125 mM O_2) resulted in rapid re-oxidation of *c* hemes with a reduced α maximum at 552 nm (fig.1). Re-oxidation of *c*-552 occurred in 3 phases of ~0.2, 70 and 500 s representing ~58, 26 and 16% of the 552 nm absorbancy, respectively. Semi-logarithmic plots of phase 1, 2 and 3 yielded first order constants (k_{obs}) of 14, 0.03 and 0.005 s^{-1}, respectively.

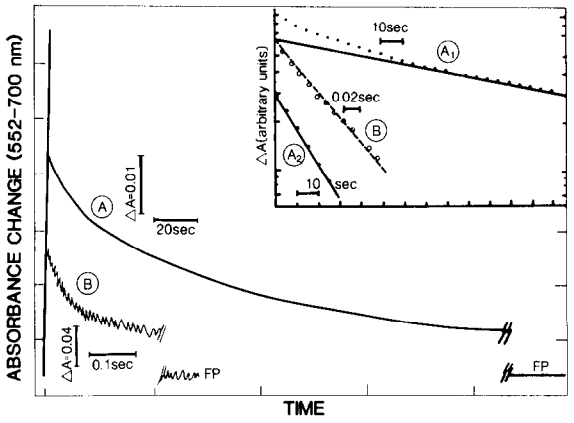


Fig.1. Kinetics of oxidation of HAO ferrous heme *c*-552 by oxygen. Change in absorbancy at 552 nm (reference 700 nm) recorded in the slow (curve A) and fast (curve B) time scale. Inset, semilogarithmic plot of data: A1, from curve A; A2, calculated by subtraction of slow-phase absorbancy change from curve A1. The final time points (FP) are 12 min.

Kinetic data for heme P460 and *c*-hemes with absorption maxima at 552 and 558 nm are summarized in table 1. The *c*-hemes with absorption maxima at ≃560 nm were reoxidized in 3 phases of rather similar length and rate constants as observed for *c*-552: phases 1, 2, 3, of 0.17, 100 and 400 s duration and rate constants 16, 0.033, 0.008 s^{-1}, respectively. In contrast to *c*-552, the majority of *c*-558 was reoxidized in the second phase (26%, 58% and 16% in phases 1, 2 and 3, respectively). Reoxidation of heme P460 was biphasic with apparent first-order rate constants of 20 and 2.5 s^{-1} for the first and second phases, respectively. Significantly, 60% of P460 reoxidation was complete in 0.17 s, corresponding in rate constant and duration of phase 1 for *c*-hemes. In sharp contrast to reoxidation of *c*-hemes, however, reoxidation of heme P460 was essentially complete in 0.4 s.

3.2. *Sequence of oxidation of P460,* c-*552 and* c-*558*

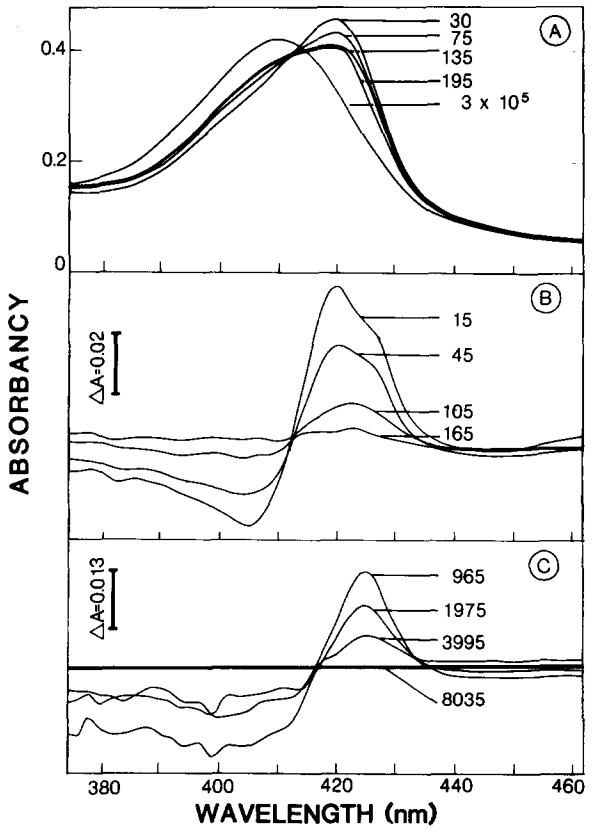
The combination of stopped-flow kinetic measurements at a fixed wavelength (fig.1, table 1) and rapid-scan measurements (fig.2) supported a tenta-

Table 1

Kinetic data for oxidation by dioxygen of ferrous hemes P460, *c*-552 and *c*-558 of HAO

Component \ Elapsed time (s)	Phase I		Phase II	Phase III
	0.17–0.2	0.4	70–100	400–500
P460 k_{obs} (s^{-1})	20	2.5		
(%)	(60)	(40)		
c-552 k_{obs} (s^{-1})	14		0.03	0.005
(%)	(58)		(26)	(16)
c-558 k_{obs} (s^{-1})	16		0.033	0.008
(%)	(26)		(58)	(16)

Values were determined as in fig.1 and section 2. The fraction reduced in each phase and duration of each phase was estimated by extrapolation to zero-time values in semi-logarithmic plots. Probable error ±20%



tive sequence of reduction for hemes P460, *c*-552 and *c*-558 (approximate molar ratios, 1:5:2). Based on the slightly greater initial rate constant, heme P460 appears to have been oxidized very early. Based on rapid-scan measurements a heme *c*-552 was the first *c*-heme to be oxidized in phase 1 with almost the same kinetics as P460 (table 1). The first *c*-heme to re-oxidize is probably a low potential heme which may be spin-coupled to P460 [12,13]. The next event simultaneous oxidation of ~2 hemes *c*-552 and a heme *c*-558 during the latter part of the rapid phase (fig.2A,B). Phase 2 appears to have involved simultaneous oxidation of 1 *c*-552 and 1 *c*-558 (fig.2C). The last heme to be oxidized was clearly a *c*-552. The latter was, in fact, sometimes extremely slow to reoxidize (~30 min).

Fig.2. Rapid-scan spectrophotometric measurement in the Soret region of the oxidation by dioxygen of ferrous *c*-hemes of HAO. Scanning time 5 ms. Numbers on the figures indicate the elapsed time after mixing (ms): (A) direct spectra including spectrum of the fully oxidized form (3×10^5 s); (B or C) differential spectra computer-calculated with reference to the final spectrum (165 or 8035 ms after mixing, respectively). Conditions as in section 2. HAO 0.18 μM (4.3 μM total heme).

3.3. *Heme P460 is the O_2-reactive site*

Heme P460 was apparently the site of reaction with O_2 as indicated by inhibition with CO of O_2-oxidation of ferrous HAO. P460 is the only CO-binding heme of the enzyme [3]. CO was bubbled for 5 min through photoreduced enzyme solution. After rapid mixing the CO and O_2 were 0.5 and 0.125 mM, respectively. Under these conditions reoxidation of *c*-552 was much slower (rapid phase 1, k_{obs} 0.06 s^{-1} as compared with 20 s^{-1} in absence of CO). The kinetics of reoxidation in presence of CO were complex and presumably reflected dissociation of CO and competition of O_2 and CO for P460. Disappearance of the HAO–P460–$Fe^2 \pm$ CO form, measured as the decrease in absorbancy at 450 nm, occurred at a very low but constantly increasing rate and required ~ 100 s for completion.

Inhibition by CO was determined with the Aminco DW-2 spectrophotometer after rapid mixing. When O_2 solution and enzyme solution containing HAO–P460–$Fe^2 \pm$CO were mixed by stopped-flow and absorption measurements made in the Union Giken instrument, photodissociation of the CO complex occurred. The phenomena results from the placement of the sample between light source and monochromator in the Union Giken spectrophotometer. The photodissociation was prevented by the use of an appropriate optical interference filter (MTO France, λ_{max} 412 nm, band pass 16 nm between light source and sample compartment. Inhibition by 0.5 mM CO was complete in the presence of the optical filter (k_{obs} 0.08 *vs* 10 s^{-1} for the enzyme in absence of CO). Removal of the filter resulted in significant prevention of CO inhibition (k_{obs} 4 s^{-1}), confirming the role of a CO-binding compound (P460). We conclude that electrons pass from *c*-hemes to P460 and then to oxygen. During oxidation of 7 *c*-hemes of HAO, heme P460 must undergo 7 cycles of reduction and oxidation.

3.4. *Product of O_2 reduction*

Because heme P460 has associated with it a reservoir of 7 ferrous *c*-hemes, an O_2 molecule may theoretically be reduced by 1, 2, 3 or 4 electrons to O_2^-, H_2O_2, H_2O + ½ O_2 or 2 H_2O, respectively. Superoxide would immediately form H_2O_2 (reviewed in [14]). Using cytochrome *c* peroxidase (CCP) as a reagent, H_2O_2 was shown to be the major product of reduction of O_2 by HAO (fig.3). The kinetics of oxidation of HAO *c*-hemes as determined at 522 nm (an isobestic point in CCP–H_2O_2 complex formation) were the same in presence or absence of CCP. Further, formation of the CCP–H_2O_2 complex measured as the increase in absorption at 438 nm (an isobestic point in the reoxidation of HAO), took place with the same initial kinetics as for *c*-heme oxidation. During the first 50 s of reaction, oxidation of ~ 14 μM HAO–heme (capable of production of ~ 7 μM H_2O_2) resulted in formation of 5.5 μM CCP–H_2O_2 complex. The results suggest that H_2O is not the major product of O_2 reduction by HAO. Rather, oxygen is reduced to O_2 and/or $H_2O_2^-$.

3.5. *Significance of O_2 reduction by HAO*

As a working model, turnover of HAO in *N*-oxygenation is thought to involve removal of electrons from N-substrates, passage of electrons via P460 to *c*-hemes, binding of O_2, passage of electrons from *c*-hemes through P460 to O_2, cleavage of oxygen and reaction of both oxygen atoms with N-compounds. Utilization of both oxygens (the absence of H_2O-production by a 'mixed-function' scheme) follows from the chemistry of ammonia oxidation: assuming that 2 e^- (+2 H^+) come from nitrogen in the $NH_2OH \rightarrow HNO$ step (and reduce O_2 to water as a part of proton pumping) there are insufficient additional valence electrons in the N-atom to allow O-addition to NH_3 (producing NH_2OH), O-addition to HNO (producing HNO_2) and also the removal of electrons for the production of water.

We suggest that the reduction of oxygen by HAO

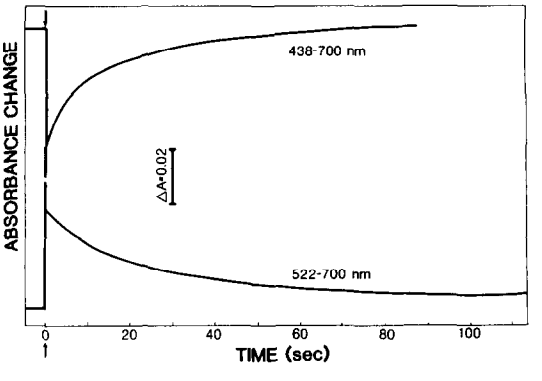


Fig.3. Simultaneous oxidation of ferrous HAO by oxygen ($\Delta A_{522-700}$) and formation of CCP–H_2O_2 complex ($\Delta A_{438-700}$).

reported here represents a part of the 2 e^- reduction and activation of O_2 for oxygenation of N-intermediate(s). The rate constant observed here for the oxidation of P460 and 4 of the 7 *c*-hemes/heme P460 in the rapid phase of the reaction with air-saturated solution is k_{obs} = 14 s^{-1}. Assuming that electrons from *c*-hemes must funnel through P460 (so that P460 is, in fact, in a transient steady-state during this phase of the reaction) this translates to a turnover number of 70 mol electron/mol P460 s^{-1}. In *Nitrosomonas*, both oxygen atoms of O_2 must be used in substrate oxygenation. As described here, HAO is capable of generating 70 mol 1 e^--reduced O/mol P460 s^{-1}. This value is of the same order of magnitude as the turnover number of HAO in vitro or in vivo (120 mol NH_2OH/mol P460 s^{-1} [2]).

ACKNOWLEDGEMENTS

We thank Dr P. Maurel for helpful discussions, Dr T. Yonetani for the CCP, Dr J.M. Lhoste for the DALF, Ms K. Terry for preparation of pure HAO and Ms C. Lyman for growth of bacteria. This work was supported by grants from the National Science Foundation (PCM-8008710); NATO (RG/136/81): INSERM and DGRST (Action spécifique no. 81.7.0424).

REFERENCES

[1] Terry, K.R. and Hooper, A.B. (1981) Biochemistry 20, 7026–7032.
[2] Hooper, A.B., Maxwell, P.C. and Terry, K.R. (1978) Biochemistry 17, 2984–2989.
[3] Lipscomb, J.D., Anderson, K.K., Münck, E., Kent, T.A. and Hooper, A.B. (1982) Biochemistry, in press.
[4] Hooper, A.B. and Terry, K.R. (1977) Biochemistry 16, 455–459.
[5] Hooper, A.B., Terry, K.R. and Maxwell, P.C. (1977) Biochim. Biophys. Acta 462, 141–152.
[6] Hooper, A.B. and Terry, K.R. (1979) Biochim. Biophys. Acta 571, 12–20.
[7] Bonnett, R. (1981) Essays Biochem. 17, 1–51.
[8] Massey, V. and Hemmerich, P. (1977) J. Biol. Chem. 252, 5612–5614.
[9] Yonetani, T. (1977) Methods Enzymol. 10, 336–339.
[10] Markley, J.L., Travers, F. and Balny, C. (1981) Eur. J. Biochem. 120, 477–485.
[11] Hiromi, K. (1979) in: Kinetics of Fast Enzyme Reactions, Theory and Practice, Kodansha, Tokyo/Wiley, New York.
[12] Vickery, L.E. and Hooper, A.B. (1981) Biochim. Biophys. Acta 670, 291–293.
[13] Lipscomb, J.D. and Hooper, A.B. (1982) Biochemistry, in press.
[14] Sawyer, D.T. and Bard, A.J. (1981) in: Oxygen and Oxy-Radicals in Chemistry and Biology (Rodgers, M.A.J. and Powers, E.L. eds) pp. 15–40, Academic Press, New York.