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A Distal Pocket Leu Residue Inhibits the Binding of O_2 and NO at the Distal Heme Site of Cytochrome *c*′

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Supporting Information

ABSTRACT: Cytochromes *c*′ are pentacoordinate heme proteins with sterically hindered distal sites that bind NO and CO but do not form stable complexes with O_2. Removal of distal pocket steric hindrance via a Leu→Ala mutation yields favorable O_2 binding (K_d ~49 nM) without apparent H-bond stabilization of the Fe−O_2 moiety, as well as an extremely high distal heme-NO affinity (K_d ~70 fM). The native Leu residue inhibits distal coordination of diatomic ligands by decreasing k_{on} as well as increasing k_{off}. The connection between distal steric constraints, k_{off} values, and distal to proximal heme-NO conversion is discussed.

The binding of diatomic gases at heme cofactors is crucial to the function of many heme proteins including globins and heme-based gas sensors. The mechanisms by which heme proteins enhance or exclude diatomic ligands have attracted considerable attention. For example, in myoglobin (Mb), a distal pocket (His) H-bond increases the affinity for O_2 relative to CO by selectively lowering the O_2 off rate constant, $k_{off}(O_2)$.[1] Conversely, discrimination against O_2 in the Heme Nitric oxide/Oxygen H-NOX domains of facultative aerobes, as well as the mammalian NO receptor soluble guanylate cyclase (sGC), has been ascribed to the absence of distal H-bond donors,[2] as well as to a weak Fe−His proximal bond.[3] Here we show that a single distal Leu→Ala replacement in cytochrome *c*′ transforms heme-O_2 reactivity from that of no detectable oxy complex to an O_2 affinity greater than that of Mb. Increased O_2 affinity stems directly from the removal of distal steric hindrance, rather than from variations in distal H-bonding or proximal ligand bond strength. The Leu→Ala replacement also traps NO at the distal heme site by boosting k_{on} and decreasing k_{off}, supporting previous suggestions that the unique distal to proximal heme-NO conversion in wild type (WT) cytochrome *c*′ is driven by steric constraints.[4,5]

Cytochromes *c*′ are mono-His ligated bacterial heme proteins that protect against nitrosative stress,[6−8] a role that can have medical importance in the protection of the pathogen *Neisseria gonorrhea* from defensive host-generated NO.[8] Their hydrophobic distal heme pockets contain a nonpolar residue (Leu, Phe, or Met) close to the Fe which significantly hinders distal ligand coordination. Consistent with severe steric restrictions at the distal site, NO binding to the distal site of *Alcaligenes xylosoxidans* cytochrome *c*′ (AXCP) is ~10^4-fold slower than the near-diffusion controlled reaction in unhindered sites such as chelated protoheme (Table 1). Moreover, unfavorable steric interactions between distally bound NO and Leu16 in AXCP have been implicated in the conversion of its six-coordinate (6c) heme-NO complex to a unique proximal five-coordinate (5c) heme-NO end product via a dinitrosyl intermediate.[4,5] The presence of Leu16 was recently shown to lower the CO affinity ~10^8-fold relative to the L16A variant, a property attributed to the energy costs of conformational changes required for CO binding at the distal site.[9]

To investigate how the steric bulk of Leu16 affects heme-O_2 and NO reactivity, we compared the reactivity of native and L16A AXCP using Resonance Raman (RR) spectroscopy and UV−vis stopped-flow kinetics. Whereas heme-O_2 coordination has not been reported for any cytochrome *c*′, L16A AXCP readily forms an oxy complex (λ_{max} 415, 537, and 571 nm (Figure S1)). Excitation at 413.1 nm enhances porphyrin RR bands at 1374 cm^{-1} (ν_4), 1504 cm^{-1} (ν_3), 1593 cm^{-1} (ν_2), and 1635 cm^{-1} (ν_{10}) characteristic of 6c low-spin heme (Figure S2). Substitution of $^{16}O_2$ with $^{18}O_2$ identifies the Fe−O_2 stretching vibration, ν(Fe−O_2) at 572 cm^{-1} from its −24 cm^{-1} isotope shift (Figure 1). As is typical of heme-oxy proteins, no ν(O−O) RR vibration was detected in the 1000−1200 cm^{-1} region.[10] The reaction of Fe^{2+} L16A with O_2 is monophasic (Figure S1). A plot of pseudo-first-order rate constants, k_{obs}, against O_2 concentration yields $k_{on}(O_2)$ = 3.5 (±0.1) × 10^6 M^{-1} s^{-1} (Figure S3, Table 1). Measurements of $k_{off}(O_2)$ using dithionite as an O_2-scavenger in the presence of CO yielded k_{off} = 0.17 ± 0.1 s^{-1} (Figure S4). The K_{dO2} value ~49 nM (calculated from k_{off}/k_{on}) shows that O_2 binding in L16A AXCP is more favorable than in Mb (Table 1). The L16A-O_2 absorbance remained stable for 3−5 h at room temperature. Over a period of ~2 days, the Soret intensity diminished and shifted from 415 to 417 nm, with a weak band evident at ~630 nm (Figure S5). Since Fe^{3+} L16A exhibits a different absorbance spectrum (Figure S5), it appears that the aging of oxy L16A is not simply autoxidation.

In contrast to the L16A variant, native AXCP autoxidizes to the Fe^{3+} state without any detectable oxy complex (Figure S6). The rate of autoxidation (k_{ox} ~14 h^{-1}) is similar to that of

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Table 1. Kinetic Parameters for Hexacoordinate Ferrous Heme Complexes with Diatomic Gases

	O_2			CO			NO			
Heme	k_{on} μM^{-1} s^{-1}	k_{off} s^{-1}	K_d nM	k_{on} μM^{-1} s^{-1}	k_{off} s^{-1}	K_d nM	k_{on} μM^{-1} s^{-1}	k_{off} s^{-1}	K_d nM	K_{dO2}/K_{dCO}
AXCP (WT)[a]	*n.o.*[b]	–	–	0.000 10	0.028	280 000	0.043	0.0060	140	–
AXCP, (L16A)[c]	3.5	0.17	49	1.1	3.7×10^{-6}	0.0034	2.9	2×10^{-7}	0.000 07	15 000
Mb (WT)[d]	17	15	880	0.51	0.019	37	22	0.000 10	0.0045	24
Mb (H64L)[d]	98	4100	42 000	26	0.024	0.92	190	0.000 10	0.000 53	45 000
Chelated protoheme[e]	62	4200	67 000	11	0.025	2.3	180	0.000 29	0.0016	29 000

[a] O_2 data from this study, NO and CO data from refs 4 and 9. [b] *n.o.*: no observable oxy complex (weak affinity). [c] O_2 and NO data from this study, CO data from ref 9. [d] Data from refs 11 and 12. [e] Data from ref 15.

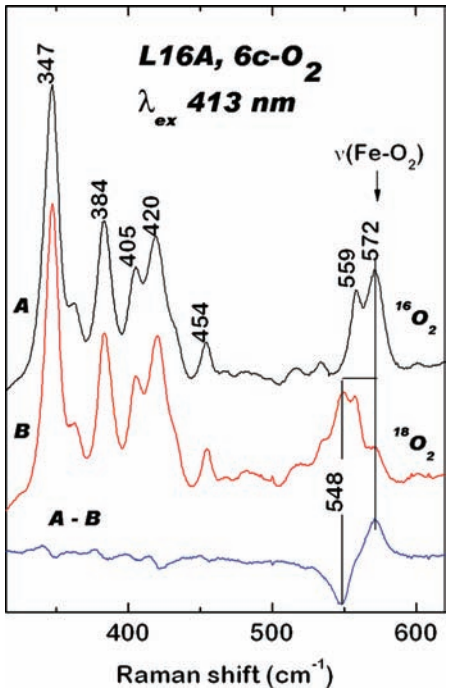

Figure 1. Low-frequency RR spectra of the heme-oxy complex of L16A AXCP prepared with $^{16}O_2$ (A) and $^{18}O_2$ (B), together with the $^{16}O_2 - {}^{18}O_2$ difference spectrum.

apolar distal Mb variants, e.g. H64L (k_{ox} ~10 h^{-1}), and much faster than that in WT Mb (k_{ox} ~0.055 h^{-1}).[11] The O_2-binding parameters of native AXCP can be inferred from previous CO and NO binding data together with reactivity trends in other heme proteins (Table 1). Because k_{on} values for O_2 and NO tend to be of similar magnitude for a given heme protein,[12] we predict $k_{on}(O_2)$ ~10^4 M^{-1} s^{-1} since k_{on}(NO) is 4.3×10^4 M^{-1} s^{-1}. Since heme complexes lacking distal H-bonding (such as AXCP) typically exhibit K_{dO2}/K_{dCO} ratios of ~10^4 (Table 1),[1] the K_{dCO} for native AXCP (~0.28 mM) points to an extremely large $K_{dO2} \geq 1$ M, which in turn implies $k_{off}(O_2)$ ~10^4 s^{-1}. The extremely weak O_2 affinity, driven by steric restrictions at the distal face from Leu16, accounts for the lack of a detectable oxy complex. Our results are consistent with previous computational studies suggesting that Leu16 significantly lowers AXCP O_2 affinity.[13]

Why does replacement of Leu16 by the less bulky Ala cause such a dramatic change in AXCP-O_2 reactivity? The higher k_{on} for the L16A variant is consistent with a more accessible distal site. However, the relatively low $k_{off}(O_2)$ of the L16A variant is surprising given that there are no distal H-bond donor side chains to stabilize heme-bound O_2.[9] While it is possible that distal H-bonding could occur via nonbonded water molecules, our kinetic data strongly argue against this. Since H-bond stabilization of heme-bound O_2 is inherently greater than that for CO, the K_{dO2}/K_{dCO} ratio provides a gauge of distal H-bonding interactions.[1] For example, WT Mb (single distal His donor) has K_{dO2}/K_{dCO} ~24, whereas the H64L Mb variant (no distal H-bonding) has a much higher ratio of ~45 000, close to that of protoheme (~29 000) (Table 1). The relatively high K_{dO2}/K_{dCO} ~15 000 for L16A AXCP therefore indicates no significant H-bonding interactions with the Fe–O_2 moiety. Neither is there evidence that $k_{off}(O_2)$ is boosted by a weak proximal Fe–His bond as recently proposed for sGC,[3] since L16A AXCP has a relatively high ν(Fe–His) frequency of 230 cm^{-1} similar to that of WT (231 cm^{-1}) (Figure S7).

To compare the effect of the L16A mutation on reactivity with other distal ligands we also measured the kinetics of NO binding. The L16A variant forms a stable 6c heme-NO complex[9] without the subsequent proximal 5c-NO conversion displayed by native AXCP. Formation of the 6c-NO species in L16A AXCP ($k_{on} = 2.9 \times 10^6$ M^{-1} s^{-1}) is ~100-fold faster than in WT protein (Table 1), consistent with less steric hindrance in the variant. Measurements of NO release from L16A (using dithionite as NO-scavenger in the presence of CO) revealed an extremely slow reaction, with only ~20% of the NO released after 19 days (Figure S9). An exponential fit of the time course (constrained to the final predicted absorbance value) yielded k_{off} ~2×10^{-7} s^{-1}, suggesting k_{dNO} ~7×10^{-14} M (Table 1). Relative to the WT protein, L16A k_{off} values for NO, O_2, and CO distal ligands are all ~10^4-fold lower. Since WT AXCP has k_{off} values similar to those for protoheme (Table 1), one interpretation is that the ligand release process in WT AXCP resembles that of unhindered heme but is dramatically inhibited by the L16A mutation. On the other hand, a recent structural, potentiometric, and theoretical study of CO binding to AXCP provided strong evidence that k_{off} in the WT protein is boosted relative to L16A by severe distal steric constraints which increase the energy of the WT-CO adduct (vide infra).[9] While Leu16 does not discriminate between diatomics at the distal site (K_{dO2}/K_{dCO} and K_{dCO}/K_{dNO} ratios for WT AXCP are similar to those of unhindered hemes), an ability to boost distal off rates may facilitate distal to proximal heme-NO conversion by causing the dinitrosyl intermediate (involved in the proximal NO binding mechanism)[4] to lose its distal NO ligand more

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rapidly than the proximal NO to yield the exclusive proximal 5c heme-NO complex in the WT-NO crystal structure.[5,14]

A recent crystallographic and physicochemical study ascribed the ~10^8-fold decrease in the CO affinity of WT AXCP relative to L16A to the energy costs of conformational changes induced by the presence of a linear Fe−C−O (170 ± 8°) geometry at the distal site, including displacement of Leu16 and a distal to proximal propionate flip.[9] The ~10^4-fold boost in k_{off}(CO) was ascribed to the CO-induced conformational changes creating a high energy state, analogous to a "loaded spring" associated with an energy differential of 57.9 kJ mol^{-1} between the WT and mutant proteins. By contrast, the L16A variant, which does not exhibit these conformational changes or energy costs, exhibits essentially irreversible CO-binding due to an unusually low k_{off}(CO) = 3.7 × 10^{-6} s^{-1}. The extent to which bent Fe−X−O geometries interact with Leu16 to induce conformational changes and/or energy costs is an ongoing question. An additional bent Fe−C−O (158°) conformer, observed only in the WT AXCP-CO crystal structure at 100 K, exhibits only a relatively small Leu16 displacement and no propionate flip.[9] On the other hand, a flipped propionate was observed in the crystal structure of the distal 5c-NO population of the R124A AXCP variant (Fe−N−O angle of 146°).[14] A fuller understanding of distal ligand discrimination in AXCP must await additional characterization of the WT distal 6c-O_2 and/or 6c-NO complexes, the relative instabilities of which have so far precluded crystallographic analysis. Nevertheless, our kinetic data suggest that steric crowding in AXCP also boosts k_{off} for heme-O_2 and heme-NO moieties which typically adopt bent Fe−X−O geometries.

The current study illustrates the dramatic effects of distal steric constraints on the binding of diatomic ligands in cytochrome *c*′. In contrast to native AXCP, the L16A distal pocket variant (which has a more accessible distal site) exhibits some of the highest known ligand affinities of any heme protein. As well as avidly binding O_2 (K_d ~49 nM) and NO (K_d ~70 fM), the overexpressed L16A variant has been shown to be isolated as the CO complex due to its picomolar CO affinity.[9] Such trapping of distal ligands is prevented in native AXCP due to steric constraints from the presence of Leu16, lowering distal ligand affinities by a factor of ~10^6−10^8 (including an ~10^4 higher k_{off}). Although Leu16 does not discriminate between diatomics at the distal site, its presence may facilitate distal to proximal heme-NO conversion in WT AXCP by promoting the release of distal NO from the dinitrosyl precursor of the proximal 5c heme-NO adduct.

■ ASSOCIATED CONTENT

Supporting Information

Experimental section and additional RR, kinetic, and absorption data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Olson, J. S.; Phillips, G. N. Jr. *J. Biol. Inorg. Chem.* **1997**, *2*, 544−552.
(2) (a) Boon, E. M.; Huang, S. H.; Marletta, M. A. *Nat. Chem. Biol.* **2005**, *1*, 53−59. (b) Weinert, E. E.; Plate, L.; A., W. C.; Olea, C.; Marletta, M. A. *Angew. Chem., Int. Ed.* **2010**, *49*, 720−723.
(3) Makino, R.; Park, S.; Obayashi, E.; Iizuka, T.; Hori, H.; Shiro, Y. *J. Biol. Chem.* **2011**, *286*, 15678−15687.
(4) Pixton, D. A.; Petersen, C. A.; Franke, A.; van Eldik, R.; Garton, E. M.; Andrew, C. R. *J. Am. Chem. Soc.* **2009**, *131*, 4846−4853.
(5) Lawson, D. M.; Stevenson, C. E. M.; Andrew, C. R.; George, S. J.; Eady, R. R. *Biochem. Soc. Trans.* **2003**, *31*, 553−557.
(6) Anjum, M. F.; Stevanin, T. M.; Read, R. C.; Moir, J. W. B. *J. Bacteriol.* **2002**, *184*, 2987−2993.
(7) Choi, P. S.; Grigoryants, V. M.; Abruña, H. D.; Scholes, C. P.; Shapleigh, J. P. *J. Bacteriol.* **2005**, *187*, 4077−4085.
(8) Turner, S. M.; Moir, J. W. B.; Griffiths, L.; Overton, T. W.; Smith, H.; Cole, J. A. *Biochem. J.* **2005**, *388*, 545−553.
(9) Antonyuk, S.; Rustage, N.; Petersen, C. A.; Arnst, J. L.; Heyes, D. J.; Sharma, R.; Berry, N.; Scrutton, N. S.; Eady, R. R.; Andrew, C. R.; Hasnain, S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 15780−15785.
(10) Mukai, M.; Savard, P.-Y.; Ouellet, H.; Guertin, M.; Yeh, S.-R. *Biochemistry* **2002**, *41*, 3897−3905.
(11) Springer, B. A.; Sligar, S. G.; Olson, J. S.; Phillips, G. N. Jr. *Chem. Rev.* **1994**, *94*, 699−714.
(12) (a) Eich, R. F.; Li, T.; Lemon, D. L.; Doherty, D. H.; Curry, S. R.; Aitken, J. F.; Matthews, A. J.; Johnson, K. A.; Smith, R. D.; Phillips, G. N. Jr.; Olson, J. S. *Biochemistry.* **1996**, *35*, 6976−6983. (b) Eich, R. F. PhD dissertation, Rice University, 1997.
(13) Martí, M. A.; Crespo, A.; Capece, L.; Boechi, L.; Bikiel, D. E.; Scherlis, D. A.; Estrin, D. A. *J. Inorg. Biochem.* **2006**, *100*, 761−770.
(14) Hough, M. A.; Antonyuk, S. V.; Barbieri, S.; Rustage, N.; McKay, A. L.; Servid, A. E.; Eady, R. R.; Andrew, C. R.; Hasnain, S. S. *J. Mol. Biol.* **2011**, *405*, 395−409.
(15) Tani, F.; Matsu-ura, M.; Ariyama, K.; Setoyama, T.; Shimada, T.; Kobayashi, S.; Hayashi, T.; Matsuo, T.; Hisaeda, Y.; Naruta, Y. *Chem.—Eur. J.* **2003**, *9*, 862−870.