Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 94-100

THE OXYGENATED FLAVOHAEMOGLOBIN FROM *ESCHERICHIA COLI*: EVIDENCE FROM PHOTODISSOCIATION AND RAPID-SCAN STUDIES FOR TWO KINETIC AND SPECTRAL FORMS

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Received July 15, 1992

SUMMARY The kinetics of dissociation and reassociation of the oxygenated species of *Escherichia coli* flavohaemoglobin (Hmp) were studied using stopped-flow rapid-scan and flash photolysis spectrophotometry at 25°C. The oxygenated compound(s) form rapidly on mixing oxygen with the NADH-reduced flavohaemoglobin. On exhaustion of NADH, with residual oxygen, decay occurs in two phases to give a form in which haem *b* and flavin are oxidized. Spectral changes during this process suggest a direct release of O_2^- from the oxy form. Photodissociation of the oxygenated species generates the unliganded protein, which recombines with oxygen to give two spectrally and kinetically distinct forms. The reversibility of the oxygen reaction and the rapid reassociation kinetics after photodissociation confirm the haemoglobin-like features of this protein.

Escherichia coli synthesizes a soluble haemoglobin-like flavohaemoprotein (Hmp), product of the *hmp* gene (1). This soluble 44 kDa protein contains a high-spin b-type haem which binds CO in the reduced state. The purified protein (2) also contains FAD, consistent with the identification of a C-terminal domain in the sequence, homologous to that in members of a family of flavoprotein oxidoreductases (3) and the flavin domain of yeast haemoglobin (4). The *E. coli* flavohaemoglobin, when reduced by NAD(P)H under aerobic conditions (2), forms an oxygenated compound with spectral characteristics similar to the corresponding compounds of yeast haemoglobin (5) and *Vitreoscilla* haemoglobin (6), with which the N-terminal haem domain of Hmp is highly homologous (1).

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0006-291X/92 $4.00

In the belief that the conserved haem domain of the flavohaemoglobin confers oxygen-binding characteristics on the protein, and is critical for protein function, we have conducted a study of the formation and dissociation of the oxygenated flavohaemoglobin from *E. coli*. Two kinetically and spectrally distinct oxygenated compounds are revealed, which exhibit dissociation and reassociation behaviour similar to the oxygenated species of the haemoglobins of *Vitreoscilla*, plants (leghaemoglobin) and animals.

MATERIALS AND METHODS

Escherichia coli strain RSC521 (RSC49/pPL341) was grown aerobically in glucose-supplemented LB medium; this strain over-produces the Hmp protein expressed from the cloned *hmp* gene (1,2). The protein was purified from cells disrupted in a French press (2). The orange-red soluble fraction obtained after removal of cell debris and membranes by centrifugation was used as starting material for a two-step purification using DEAE-Sepharose CL6B and Sephacryl S200 as described previously (2). The protein was stored and used in a buffer that contained 50 mM NaCl, 50 mM Tris-HCl, pH 8.0.

Stopped-flow rapid-scan spectrophotometry was performed using apparatus similar to that described previously (7,8). The present apparatus (Orii and Nagamura, in preparation) records 512 absorbance spectra over 208 nm with a maximal scan rate of 208 nm/1.04 ms. Data on the main memory of a computer were saved on a magneto-optical storage device for subsequent processing.

The protein was diluted so that the final concentration in the observation cell was about 3.3 μM. Reduction was achieved in a reservoir of the stopped-flow apparatus by adding NADH to 0.7 mM, and incubation under a flow of nitrogen gas for 10 min. Buffer in the second reservoir was air-saturated; all experiments were carried out at 25°C. Photolysis of the oxygenated form was achieved by a flash from a Rhodamine 590 dye laser (250 mJ, rated duration 300 ns). Absorption spectra were recorded on the same apparatus, but by firing a xenon flash between 1 μs and 500 μs after photolysis.

RESULTS AND DISCUSSION

On mixing NADH-reduced Hmp with air-saturated buffer in the stopped-flow apparatus, extensive formation of the oxygenated compound (a 415 nm peak in the absolute spectrum, not shown) occurred during the dead-time of the apparatus. Rapid-scan studies in the visible region (not shown) revealed corresponding peaks at 544 nm and 580 nm in accordance with our previous paper (2). The oxy form was stable for about 250 s under these conditions; on exhaustion of NADH (as indicated by near-UV absorbance decrease in the difference spectra of Fig.1A), the oxyform decayed to an oxidized species. The rate of oxygen consumption was estimated to be 0.5 μM O_2 s^{-1} (130 μM O_2 consumed in 260 s). Table 1 summarizes the relaxation times derived from the traces at varied

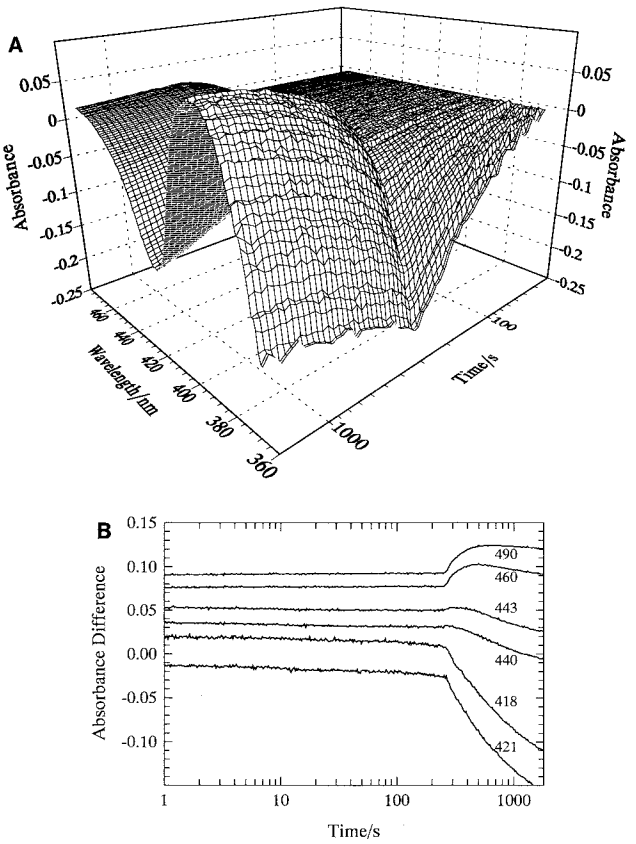

Fig.1. Decay of the oxygenated species of *E. coli* flavohaemoglobin. The protein in 50 mM NaCl, 50 mM Tris-HCl, pH 8, and reduced with NADH (0.7 mM) was allowed to react with air-saturated buffer in the stopped flow rapid-scan apparatus at 25°C. Concentrations of the protein and oxygen after mixing were approximately 3.3 μM and 130 μM, respectively. The 3-dimensional display (A) shows difference spectra recorded with the 36-s spectrum as reference. The absorbance changes after 1 s (B) are plotted on a logarithmic time scale. Traces in (B) have been displaced on the absorbance axis for clarity.

wavelengths. The traces at 418, 421 and 460 nm (Fig. 1B) yielded two relaxation times, suggesting occurrence of two sets of spectral changes.

The first phase, accounting for 40% of the total, is shown in Fig.2A. The decay of the oxyform (a trough at 421 nm in difference spectra with the 259-s spectrum as reference) was accompanied by simultaneous oxidation of FAD (460 nm; $1/\tau$=0.013 s^{-1}) and an absorbance increase at 490 nm ($1/\tau$=0.012 s^{-1}). The latter is attributed to oxidation of haem to the high-spin Fe(III) state (2); concomitant appearance of a peak around 385 nm suggests that ferric haem as well as oxidized FAD is the product. On the other hand, the traces at 440 and 443

Table 1. Relaxation times derived at various wavelengths following the reaction of NADH-reduced Hmp with oxygen and subsequent exhaustion of NADH

λ (nm)	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	r^2
417.7	0.0109	0.0016	0.9994
421.0	0.0073	0.0014	0.9994
439.7	-	0.0014	0.9959
442.9	-	0.0018	0.9957
459.6	0.0128	0.0015	0.9939
489.7	0.0120	-	0.9977

The signals are attributed mainly to the oxygenated species (417.7, 421 nm), flavin (459.6 nm) and ferric high-spin haem (489.7 nm). r^2 signifies coefficient of determination in the fitting.

nm are almost monophasic. Since the isosbestic point for the initial spectral change is at 443 nm, these traces must represent the second phase of decay of the oxy form which accounts for 60% of the total change. Fig.2B indicates that the

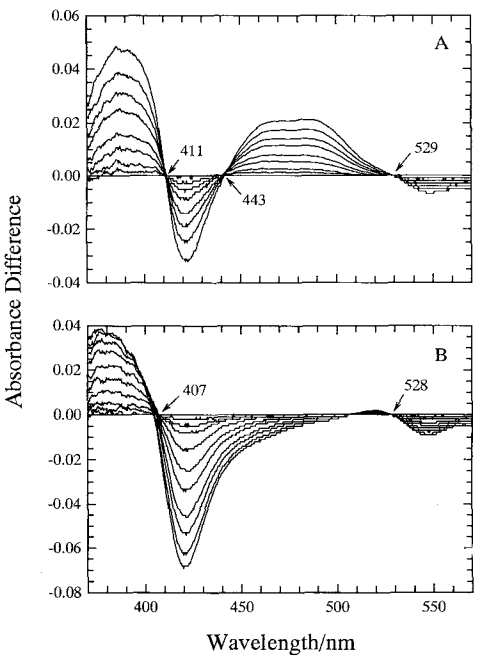

Fig.2. Difference spectra on depletion of NADH following the reaction between reduced flavohaemoprotein and oxygen. A, difference spectra recorded with the 259-s spectrum as reference. Times of recording are, in the order of decreasing absorbance at 421 nm, 259, 264, 270, 275, 281, 293, 305, 324, and 352 s. B, difference spectra recorded with the 498-s spectrum as reference. Times of recording are, in the order of decreasing absorbance at 421 nm, 498, 508, 530, 552, 611, 705, 814, 1019, 1200, 1503, and 1807 s. Conditions were as in Fig.1.

decay of the oxy form to a ferric species in the second phase is monotonous without accompanying the absorbance increase at 490 nm. Instead, in the difference spectra, the 421 nm trough is flanked by a valley tailing to a longer wavelength, explaining a decline of the 460 nm trace after about 500 s with $1/\tau$ =0.0015 s^{-1} in Fig. 1B. This may be ascribed to re-reduction of FAD by O_2^- (see below).

Unless we assume existence of an oxidase of unknown nature different from Hmp in the present preparation, and this is most unlikely, we are tempted to assume that Hmp directly participates in the reduction of molecular oxygen. Predominance of the oxy form before exhaustion of NADH and its decay into the ferric species upon NADH exhaustion suggests that in the latter process O_2^- is released yielding ferric Hmp. This mechanism of oxygen reduction is reminiscent of the O_2^- generating system in neutrophils in which NAD(P)H-reduced cytochrome *b*-558 directly reduces molecular oxygen yielding O_2^- (9). Thus, although it is expected, and should be confirmed by further studies, that O_2^- is disproportionated immediately, it is likely that O_2^- also acts on oxidized FAD to reduce it as speculated above. Assuming that all Hmp (3.3 mM) is involved in the oxygen reduction, the rate of oxygen consumption estimated above, 0.5 mM O_2 s^{-1}, gives an apparent turnover number of 0.15 s^{-1}. Since this is much higher than $1/\tau_1$ (0.0128 s^{-1}, maximal), the proposed O_2^- release mechanism may not be able to explain the oxidase activity during the steady state. Thus, further studies are necessary in this respect.

Unlike neutrophil cytochrome *b*-558, Hmp clearly forms an oxygen compound like oxyhaemoglobin and oxymyoglobin as evidenced by a characteristic absorption spectrum in the visible region (2). This similarity was further supported by its reversible photodissociation. Laser irradiation of the oxy-form in the steady state after mixing (Fig.1) elicited the spectral changes shown in Fig.3, which are difference spectra recorded 20 μs or 500 μs after the laser flash with the 1 μs post-photolysis spectrum as reference. After 500 μs, the difference spectrum shows a peak at 413 nm, a trough at 440 nm, and a distinct shoulder at about 425 nm. The peak and trough positions in the difference spectrum are in good agreement with those in a difference spectrum between a sample scanned during the oxygenated steady state (not shown) and a sample at 1800 s (deoxy), and with the CO-reduced minus reduced difference spectrum (2). We conclude, therefore, that the major spectral change in Fig.3 is due to photodissociation of an oxygenated form of the flavohaemoglobin with spectral characteristics (in the Soret

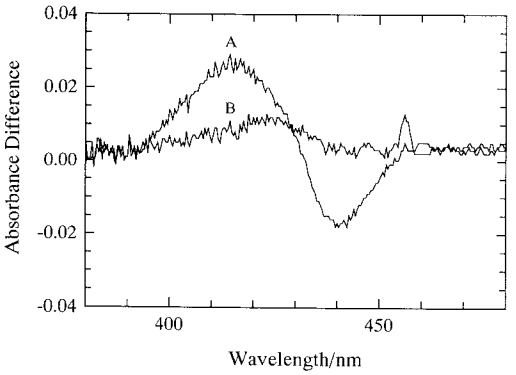

Fig.3. Photolysis of oxygenated flavohaemoglobin. Oxygenated flavohaemoglobin was prepared as in Fig.1 except that the NADH concentration was 1 mM before mixing with aerated buffer in the flow-flash apparatus. The sample was photolysed with a laser flash and time difference spectra were recorded at 25°C. A, 500 μs minus 1 μs. B, 20 μs minus 1 μs.

region) similar to, but not identical with, the carbonmonoxy form. However, the extent of photolysis was 12% of that expected in a static difference spectrum (oxygenated minus deoxy), indicating that the quantum yield of photodissociation of oxyflavohaemoglobin is much less than 1. The time difference spectrum recorded at 20 μs minus 1 μs (Fig.3) reveals heterogeneity in the recombination event. The more rapidly formed, but minor, oxygenated compound has an absorbance maximum at 425 nm. It is to be noted that this fast binding occurs in the same time range as for *Vitreoscilla* haemoglobin (6), leghaemoglobin (10), and cytochrome *c* oxidase (7).

The existence of two oxygenated states of *E. coli* flavohaemoglobin observed in our photolysis studies and after exhaustion of reductant is consistent with data (to be published elsewhere) which show that reduction by NADH in the presence of oxygen is also kinetically heterogenous. Epr studies at the pH used here (pH 8.0) reveal high-spin haem signals with both rhombic and axial symmetry (2), and these species may explain the heterogeneity of the oxy forms. Although the significance of this heterogeneity is not understood at present, the results highlight analogies in ligand association and dissociation with established haemoglobins and suggest a crucial functional role for the relatively stable oxygenated species.

ACKNOWLEDGMENTS

RKP thanks the SERC for a Research Grant (GR/G/13761) and the Royal Society for a Leverhulme Trust Senior Research Fellowship and a study visit grant for

Japan. This work was supported in part from a Grant-in-Aid for Scientific Research on Priority Areas of "Cellular Energy" (No. 04266105) to YO from the Ministry of Education, Science and Culture, Japan. We are grateful to Simon Andrews, John Guest, and Austen Riggs for sight of papers in press.

REFERENCES

1. Vasudevan, S. G., Armarego, W. L. F., Shaw, D. C., Lilley, P. E., Dixon, N. E. and Poole, R. K. (1991) *Mol. Gen. Genet.* **226**, 49-58.
2. Ioannidis, N., Cooper, C. E. and Poole, R. K. (1992) *Biochem. J.*, accepted for publication.
3. Andrews, S. C., Shipley, D., Keen, J. N., Findlay, J. B. C., Harrison, P. M. and Guest, J. R. (1992) *FEBS Lett.* **302**, 247-252.
4. Zhu, H. and Riggs, A. F. (1992) *Proc. Natl. Acad. Sci. U.S.A.*, in the press.
5. Oshino, R., Asakura, T., Takio, K., Oshino, N. and Chance, B. (1973) *Eur. J. Biochem.* **39**, 581-590.
6. Orii, Y. and Webster, D. A. (1986) *J. Biol. Chem.* **261**, 3544-3547.
7. Orii, Y. (1984) *J. Biol. Chem.* **259**, 7187-7190.
8. Orii, Y. and Anni, H. (1990) *FEBS Lett.* **267**, 117-120.
9. Isogai, Y., Shiro, Y., Nasuda-Kouyama, A. and Iizuka, T. (1991) *J. Biol. Chem.* **266**, 13481-13484.
10. Wittenberg, J. B., Appleby, C. A. and Wittenberg, B. A. (1972) *J. Biol. Chem.* **247**, 527-531.