Kinetics and Thermodynamics of the Reaction of Deoxyhemerythrin with Nitric Oxide

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Deoxyhemerythrin reacts with NO to form a 1:1 adduct shown spectrophotometrically. The kinetics of the formation have been studied directly by stopped-flow measurements at four different temperatures (0.0–23.6 °C). The kinetics of the dissociation have been studied, also by stopped-flow techniques, at five different temperatures (4.0–35.1 °C) using three different scavengers [Fe(II)(edta)$^{2-}$, O_2 and sperm whale deoxymyoglobin], which gave similar values. For the formation $k_f = (4.2\pm0.2)\times10^6$ M^{-1} s^{-1}, $\Delta H_f^{\ddagger} = 44.3\pm2.3$ kJ mol^{-1}, $\Delta S_f^{\ddagger} = 30\pm8$ J mol^{-1} K^{-1} and for the dissociation $k_d = 0.84\pm0.02$ s^{-1}, $\Delta H_d^{\ddagger} = 95.6\pm2.1$ kJ mol^{-1}, $\Delta S_d^{\ddagger} = 74\pm7$ J mol^{-1} K^{-1} (25 °C, $I = 0.2$ M and pH 7–8.1). From the kinetic data the thermodynamic data for the formation of HrNO were calculated: $K_f = (5.0\pm0.3)\times10^6$ M^{-1}, $\Delta H^{\ominus} = -51.3\pm3.1$ kJ mol^{-1} and $\Delta S^{\ominus} = -44\pm11$ J mol^{-1} K^{-1} (25 °C). The kinetic data suggest that NO occupies the same iron(II) site in deoxyhemerythrin as oxygen does. The equilibrium constant for the formation of Fe(II)(edta)(NO)$^{2-}$ has been redetermined: $K_1 = (1.45\pm0.07)\times10^7$ M^{-1}, $\Delta H^{\ominus} = -77.5\pm1.5$ kJ mol^{-1} and $\Delta S^{\ominus} = -123\pm5$ J mol^{-1} K^{-1} (25 °C).

Hemerythrin is found in several marine invertebrates and is one of only three respiratory proteins which are known to interact reversibly with oxygen.[1] The structural aspects of the protein and of the dinuclear iron site have been thoroughly examined with a multitude of investigative probes.[1–4] Deoxyhemerythrin (abbreviated Hr°) contains an Fe(II)Fe(II) site, and is responsible for the oxygen uptake. The product, oxyhemerythrin (HrO_2), contains the active site shown in Fig. 1(A). The deoxy form is now believed to have a similar site, with one pentacoordinate iron atom to which the O_2 attaches. The kinetics of reaction of O_2 with Hr° have been thoroughly examined, particularly by flow[5,6] and temperature-jump[6] techniques, but also by a laser photolytic pertubation method.[7,8] The interaction of the anions CNO^-, N_3^- and F^- with deoxyhemerythrin is now established.[9–12] In the most recent study, the kinetics and thermodynamics of interaction of HCNO, HN_3 and HF (the anion is introduced as the undissociated acid) have been thoroughly determined.[12] We can now add NO to those ligands which interact reversible with deoxyhemerythrin.[13,14] We have therefore investigated the kinetics and thermodynamics of this interaction in order to compare them with the other data.[5,6,12] We have used the octameric form of the protein (molecular weight 108 000) from the coelomic fluid of *Phascolopsis gouldii*.

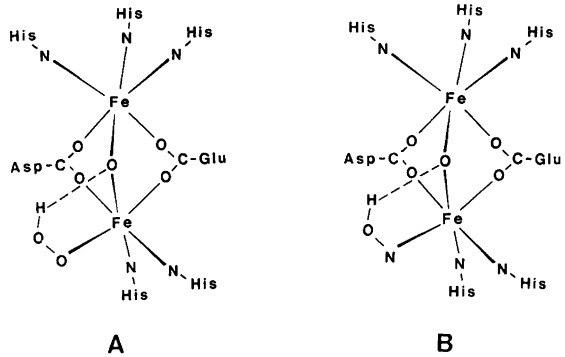

Fig. 1. (A) Proposed structure[1] for the binuclear iron site in HrO_2. (B) It seems likely from this study and spectroscopic studies[14] that NO binds at the same site as oxygen and that both structures are stabilized by an intramolecular hydrogen bond, OH---O–O and OH---O–N, respectively.

Experimental

Instruments. Kinetic data were obtained using a Dionex stopped-flow apparatus, and absorption spectra were determined using a Cary 14 spectrophotometer. Both were interfaced with an OLIS datacollecting system. Temperature-jump experiments were performed using a Durrum model D-150 apparatus. The laser experiments were made

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using a model DL-1020 dye laser (Phase-R Corp.) and a single-beam double monochromator (ISA Instruments) interfaced to a modified Compaq computer (OLIS On-line Instrument Systems, Inc.). Solutions of Coumarin C525 in methanol were used to produce an excitation flash of 100 mJ at 525 nm.

Materials. Oxyhemerythrin was isolated from the coelomic fluid of worms of the species *Phascolopsis gouldii*, which were obtained live from Marine Biological Laboratories, Woods Hole, MA. Solutions of methemerythrin and deoxyhemerythrin were prepared and their concentrations determined as described previously.[12,15] Deoxymyoglobin was prepared from metmyoglobin (Sigma) by reduction with dithionite as described for deoxyhemerythrin. Nitric oxide was obtained from Matheson, Inc., La Porte, Texas. All other chemicals were of analytical grade. Aqueous solutions were prepared using doubly distilled deionized water.

Preparation of solutions. For all the kinetic and thermodynamic measurements, preparation and manipulation of solutions were carried out in an argon atmosphere with scrupulous exclusion of oxygen as described previously.[12]

Saturated solutions of NO, used as stock solutions, were prepared before each series of measurements by passing NO gas first through 4 M NaOH and then degassed buffer solutions at ambient temperature and pressure. If the NO gas was not scrubbed with base, it was found that the saturated NO solutions showed absorbance at 355 nm, certainly due to NO_2^-. The concentration of NO in the stock solution was always determined prior to use by colorimetric measurements using the reaction of $Fe(II)(edta)^{2-}$ with NO as described below. The concentrations of NO in the saturated buffer solutions were typically 1.3–1.6 mM.

Most of the experiments were carried out using an 0.1 M phosphate buffer solution by dissolving 13.92 g $K_2HPO_4 \cdot 3H_2O$ and 5.31 g KH_2PO_4 in 1000 ml of water. Other buffer solutions were made following standard procedures.

Determination of nitric oxide concentration. The concentration of nitric oxide in the aqueous stock solutions was determined spectrophotometrically using the absorption of the $Fe(II)(edta)(NO)^{2-}$ complex at $\lambda = 434$ nm. Aliquots of the aqueous solutions saturated with NO (typically 0.5 ml) were added to an oxygen-free solution of 0.01 M $Na_2Fe(II)(edta)$ and 0.01 M $Na_4(edta)$ in 0.1 M phosphate buffer (typically 2.2 ml), and the absorbance at $\lambda = 434$ nm was measured as a function of the amount of NO solution added. Since the molar absorbance of $Fe(II)(edta)^{2-}$ ($\varepsilon < 5$ M^{-1} cm^{-1}) is negligible compared to that of $Fe(II)(edta)(NO)^{2-}$, the increase in absorbance can be taken as a direct measure of the concentration of $Fe(II)(edta)(NO)^{2-}$, which has $\varepsilon = 755$ M^{-1} cm^{-1} (see below).

The molar absorbance of $Fe(II)(edta)(NO)^{2-}$ at $\lambda = 434$ nm was determined by adding aliquots of 0.1 M $Na_4(edta)$ (oxygen free) to a large excess of a saturated solution of NO in 0.1 M phosphate buffer and then measuring the change in absorbance as a function of the concentration of the Fe(II). The results were consistent with quantitative formation of a 1:1 $Fe(II)(edta)^{2-}$ nitric oxide complex with a value of $\varepsilon = 755$ M^{-1} cm^{-1} at $\lambda = 434$ nm. Stock solutions used to prepare the $Fe(II)(edta)^{2-}$ solutions were prepared from either $FeSO_4 \cdot 7H_2O$ or $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, and the Fe(II) concentration was determined using a standard colorimetric method based upon the formation of the strongly red-coloured $Fe(phen)_3^{2+}$ species ($\varepsilon = 11100$ M^{-1} cm^{-1} at 508 nm).[16]

Spectral measurements. The spectrum of $Fe(II)(edta)(NO)^{2-}$ in the region 300–800 nm was obtained from a mixture of $Fe(II)(edta)^{2-}$ and NO with either in excess and by correcting for the absorbance of the species in excess or by measuring a solution containing equivalent amounts of the two

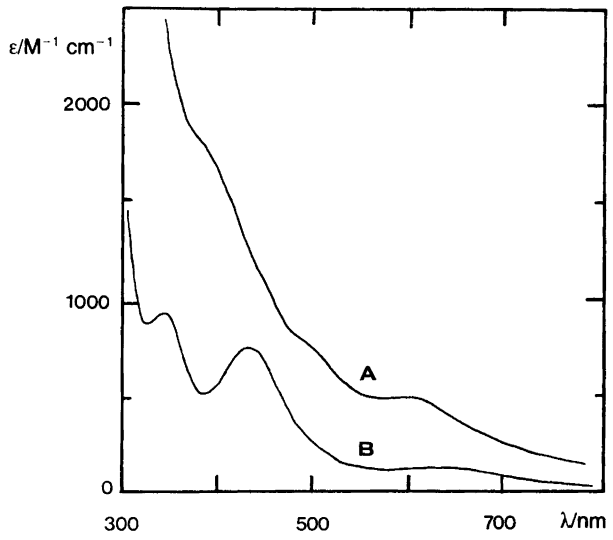

Fig. 2. Absorption spectra of HrNO (A) and $Fe(II)(edta)(NO)^{2-}$ (B).

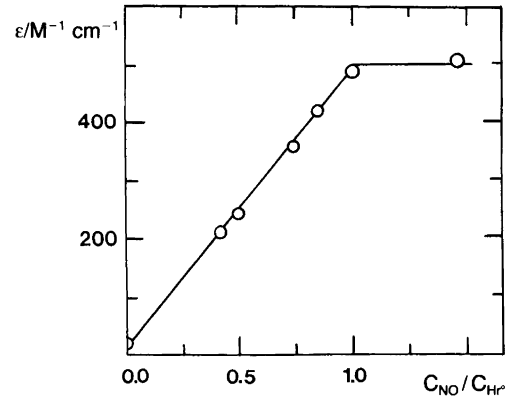

Fig. 3. The change in molar absorbance ε at 550 nm of a 5×10^{-4} M solution of Hr° as a function of added nitric oxide shows that only one NO molecule is bound per subunit.

species. This gave, within experimental error, identical spectra (Fig. 2). The spectrum of HrNO was measured in a similar manner using an excess of protein, an excess of NO or equivalent amounts with a similar result (Figs. 2 and 3).

Kinetic measurements. The dead-time of the instrument was about 6 ms, and mixing of reactant concentrations to give a reasonable absorbance change, e.g. 2×10^{-4} M Hr° and 10^{-5} M NO, resulted in almost complete reaction in the mixer. Lower concentrations of reactants yielded measureable rates but with very small changes in absorbance. This problem was solved as illustrated by the following example. Solutions of 2.8×10^{-5} M Hr° and 9.8×10^{-5} M NO were mixed and the observed absorbance became constant after ca. 30 ms. From the change in absorbance (0.007 at $\lambda = 410$ nm and path-length 1.7 cm) and the known molar absorbances of the involved species it was calculated that the part which was monitored corresponded to reaction of 3.4×10^{-6} M Hr°. The concentration of the reactants at t_0 (the time for the first observation) was then calculated to be $[Hr°]_{t_0} = 3.4\times10^{-6}$ M and $[NO]_{t_0} = 3.84\times10^{-5}$ M, i.e. pseudo-first-order conditions. The average concentration of NO during the part of the reaction which was monitored was therefore $[NO]_{av} = [NO]_{t_0} - 1/2\times[Hr°]_{t_0} = 3.67\times10^{-5}$ M. This gave an observed rate constant of 139 s^{-1} and a computed second-order rate constant $k_f = k_{obs}/[NO]_{av} = 139/(3.67\times10^{-5}) = 3.8\times10^6$ M^{-1} s^{-1}.

Calculations. All calculations were made within the framework of non-linear least-squares calculations. Pseudo-first-order rate constants were calculated from the change in absorbance with time using the expression $A = A_\infty + (A_0 - A_\infty)\exp(-k_{obs}t)$, where A_0 is the absorbance at $t = 0$ and A_∞ is the absorbance at completion. Activation parameters were calculated using the expression $k_r = (kT/h)\exp(-\Delta H^‡/RT + \Delta S^‡/R)$.

Results and discussion

Stoichiometry of the reaction of Hr° with NO. The reaction between Hr° and NO shown in eqn. (1) was studied spectrophotometrically. Fig. 3 shows the absorbance of a 5×10^{-4} M solution of Hr° as a function of the amount NO added (here and in the following the concentrations of Hr° are defined in terms of the subunits of the octamer).

$$\mathrm{Hr°} + \mathrm{NO} \underset{k_d}{\overset{\overset{K_f}{k_f}}{\rightleftharpoons}} \mathrm{HrNO} \qquad (1)$$

There is a linear change in the molar absorbance (550 nm) with increasing amount of NO for $C_{NO}/C_{Hr°} \leq 1$, followed by a constant absorbance for $C_{NO}/C_{Hr°} \geq 1$ (here and in the following C denotes stoichiometric concentrations and [] denotes actual concentrations). This shows that only one NO molecule is bound per subunit, and that for $C_{Hr°} = C_{NO} = 5\times10^{-4}$ M the formation of the nitric oxide complex is more than 95% complete. This gives a lower-limit estimate for $K_f > 8\times10^5$ M^{-1} [eqn. (1)]. It follows from these experiments that the equilibrium constant K_f could be determined only with difficulty from spectroscopic measurements of equilibrated solutions, but as described below we determined its value on the basis of kinetic data.

The spectrum of HrNO, shown in Fig. 2, exhibits three shoulders positioned at (ε/M^{-1} cm^{-1}, λ/nm) = (1671,400), (761,500) and (495,600). The shape of the absorption curve is very similar to that previously reported,[13] but the molar absorption coefficients obtained by us are significantly larger. We have no explanation for this, but note that we obtained the same molar absorption coefficients using different preparations of protein. The HrNO adduct is relatively stable at room temperature in an anaerobic atmosphere, shown by the fact that the absorption spectrum (300–800 nm) did not change significantly within 20 min. This is in agreement with a report that HrNO undergoes only slow ($t_{1/2} \approx 6$ h) auto-oxidation to form semimet [Fe(II),Fe(III)].[14]

Kinetics of the formation and dissociation of HrNO. Preliminary measurements indicated that the formation of HrNO was very rapid even at low concentrations of reactants. Therefore pertubation methods which had been succesfully used with the $Hr°/O_2$ system[5–8] were attempted. No temperature-jump relaxations of equilibrated solutions of Hr° with NO at various (low) concentrations were observed, even when 10–20 jumps were collected. This was ascribed to insufficient dissociation of HrNO even with a 15 °C jump, combined with a relatively small absorbance coefficient for the NO adduct. Another pertubation method which was also succesful for the $Hr°/O_2$ system is laser photolysis.[7,8] We found that irradiation of a 0.1 mM solution of HrNO by a 525 nm, 5 μs pulse yields no relaxations attributable to (dark) recombination of Hr° with NO, following photodissociation of HrNO. Using the same experimental setup we found that a 0.4 mM solution of Hr° in 1.3 mM O_2 gave a 119 μs relaxation half-life (25 °C), which agrees well with the known[5] formation rate constant of 7.4×10^6 M^{-1} s^{-1}. It is apparent that HrNO is much less easily photodissociated than HrO_2, which is a similar behaviour to the heme adducts, where quantum yields for sperm whale deoxymyoglobin adducts with O_2 and NO are 0.13 and ≤0.01, respectively.[17] The kinetics of the formation and dissociation of HrNO were finally determined spectrophotometrically by stopped-flow measurements. The majority of the measurements were carried out using a 0.1 M phosphate buffer (pH 7.0).

Formation. The kinetics of the formation were studied using pseudo-first-order conditions with NO usually in excess. It was not possible to vary the concentrations of the reactants within a large range: the concentration of the reactant in excess had to be sufficiently small so that the observed rate constants were within the time-scale of the

Table 1. Kinetic data for the reaction of Hr° with NO.[a]

10^5[Hr°][b] /M	10^5[NO][b] /M	k_{obs}[c] /s^{-1}	10^6k_f[d] /s^{-1}	10^6k_f(calc)[g] /s^{-1}	T /°C
4.0	0.4	174	4.4	3.8	23.6
0.1	8.6	312	3.6	3.8	23.6
0.1	5.1	212	4.2	3.8	23.6
0.2	3.7	139	3.8	3.8	23.6
0.2	2.9	105	3.6	3.8	23.6
0.2	3.0	60	2.0[e]	2.3	16.0
0.2	7.0	130	1.85[f]	1.80	12.3
0.2	3.4	56	1.65[f]	1.80	12.3
0.3	7.0	57	0.81	0.74	0.0
0.3	3.4	24.7	0.73	0.74	0.0

[a]0.1 M potassium phosphate, pH 7 and $I = 0.2$ M. [b]The concentrations are those at the half-life calculated from the stoichiometric concentrations and the observed changes in absorbances as described in the text. [c]Each value is the average of two experiments monitored at $\lambda = 410$ nm. [d]Calculated using $k_f = k_{obs}/[Hr°]$ or $k_f = k_{obs}/[NO]$. [e]At pH 8.1 using a 0.05 M Na_2SO_4, 0.05 M tris(hydroxymethyl) aminomethane buffer solution ($I = 0.2$ M). [f]The same k_{obs} values were obtained when the reaction was monitored at $\lambda = 370$ nm. [g]Calculated from the activation energies in Table 2.

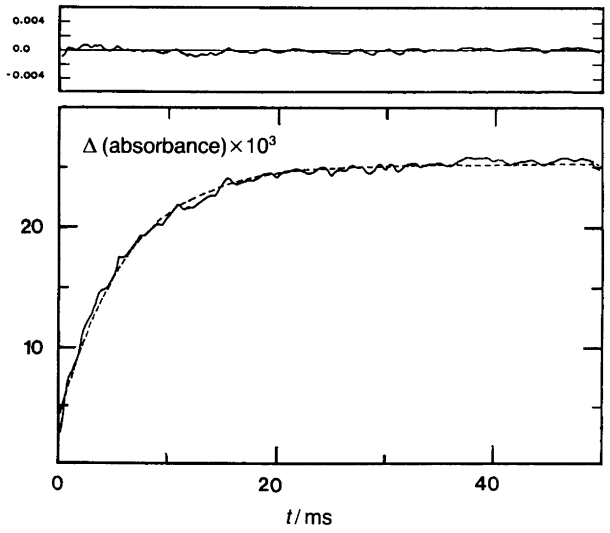

Fig. 4. Observed (——) and calculated (- - -) change of absorbance (410 nm) with time for the reaction of 4×10^{-5} M Hr° with 8×10^{-6} M NO 0.1 M phosphate (pH 7) at 23.6 °C. The upper curve shows the residual ($ABS_{calc}-ABS_{obs}$).

stopped-flow instrument, while the concentration of the reactant in deficiency had to be large enough that a measurable change in absorbance was produced. These requirement defined the variation in concentrations of the reactants as shown in Table 1. With these concentrations a change in absorbance of only 0.01–0.03 was obtained; however, by accumulating data for 6–10 consecutive runs the averaged final result gave smooth and reproducible traces of absorbance versus time (Fig. 4). These averaged traces followed a first-order expression for at least three half-lives, and the observed rate constants are shown in Table 1. The rate expression for the equilibration reaction, eqn. (1), is shown in eqn. (2), where X = Hr° or NO.

$$k_{obs} = k_d + k_f[X] \qquad (2)$$

The variation of k_{obs} with (an excess of) either [Hr°] or [NO] clearly shows that the contribution of k_d to the rate expression is less than the experimental error for the concentration regions used, and therefore we have the simplified expressions eqn. (3). Note that this interpretation also

$$\begin{aligned} k_{obs} &= k_f[Hr°] \text{ for an excess of Hr°} \\ k_{obs} &= k_f[NO] \text{ for an excess of NO} \end{aligned} \qquad (3)$$

is in agreement with the preliminary spectral data mentioned above and with the kinetic data for the dissociation, k_d, discussed later. This interpretation is also supported strongly by the fact that the same value for k_f is obtained for the experiments with either Hr° or NO in excess (Table 1). The majority of the experiments were made at $\lambda = 410$ nm, but a few made at $\lambda = 370$ nm gave the same results. A single experiment at pH 8.1 [0.05 M Na_2SO_4 and 0.05 M tris(hydroxymethyl)aminomethane] gave the same result as found for pH 7. From the temperature dependence of k_f (Fig. 5) the activation parameters were calcu-

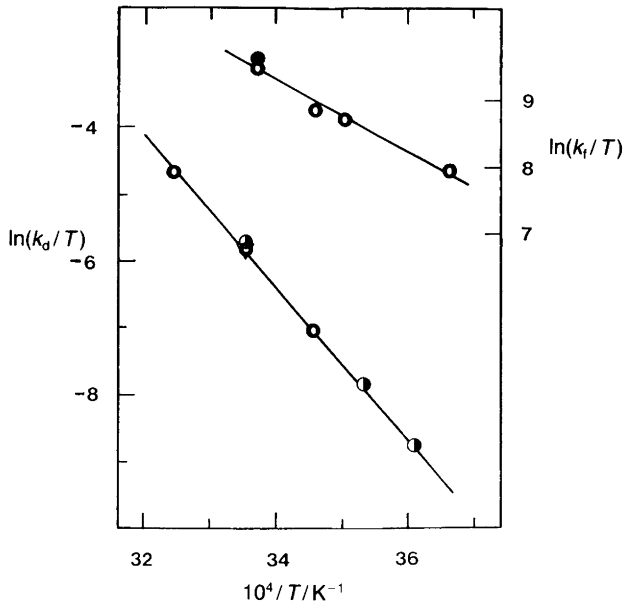

Fig. 5. The temperature dependence of the rate constants for the formation (k_f, upper right-hand line) and the dissociation (k_d, lower left-hand line) of HrNO. The formation was studied using an excess of Hr° (●) and an excess of NO (○). The dissociation was studied using Fe(II)(edta)$^{2-}$ (○), O_2 (◑) and Mb (▽) as scavengers. The solid lines represent values calculated using parameters given in Table 2.

Table 2. Kinetic and thermodynamic data for the reactions of deoxyhemerythrin with NO and other nucleophiles at 25 °C.

$$Hr^{o} + X \underset{k_d}{\overset{k_f}{\rightleftharpoons}} HrX$$

Conditions	X	$10^{-6}k_f$ /M^{-1} s^{-1}	$\Delta H^{\ddagger}$ /kJ mol^{-1}	$\Delta S^{\ddagger}$ /J mol^{-1} K^{-1}	Ref.
pH 7–8.1, I = 0.2 M	NO[a]	4.2(±0.2)	44.3(±2.3)	30(±8)	This work
pH 8.1, I = 0.015 M	O_2	7.4	34	4	5
pH 6–9, I = 0.5 M	HN_3	0.030	26	−71	12
–	HCNO	0.058	33	−29	12
–	HF	0.0050	21	−100	12
Conditions	X	k_d /s^{-1}	$\Delta H^{\ddagger}$ /kJ mol^{-1}	$\Delta S^{\ddagger}$ /J mol^{-1} K^{-1}	Ref.
pH 7–8.1, I = 0.2 M	NO[a]	0.84(±0.02)	95.6(±2.1)	74(±7)	This work
pH 8.1, I = 0.015 M	O_2	51	86	79	5
pH 6–9, I = 0.5 M	HN_3	0.1	51	−93	12
–	HCNO	0.012	58	−85	12
–	HF	0.010	52	−105	12
Conditions	X	$10^{-6}K_f$ /M^{-1}	$\Delta H^{\ominus}$ /kJ mol^{-1}	$\Delta S^{\ominus}$ /J mol^{-1} K^{-1}	Ref.
pH 7–8.1, I = 0.2 M	NO[a]	5.0(±0.3)	−51.3(±3.1)	−44(±11)	This work
pH 8.1, I = 0.015 M	O_2	0.15	−52	−75	5
pH 6–9, I = 0.5 M	HN_3	0.30	−26	19	12
–	HCNO	4.8	−25	56	12
–	HF	0.50	−32	3	12
pH 7, I = 0.2 M	NO[a,b]	14.5(±0.7)	−77.5(±1.5)	−123(±5)	This work

[a]The standard deviations for the parameters determined in this work are given in parentheses. [b]These data are for reaction with $Fe(II)(edta)^{2-}$.

lated (Table 2). There is excellent agreement between the observed and calculated rate constants (Table 1), and the activation parameters are well-defined (Table 2).

Dissociation: Fe(II)edta as scavenger. The $Fe(II)(edta)^{2-}$ species forms a 1:1 complex with NO. The absorption spectrum, shown in Fig. 2, exhibits three maxima positioned at (ε/M^{-1} cm^{-1}, λ/nm) = (939,345), (755,434) and (124,635). Previously published molar absorbances are significantly smaller[18] and larger,[19] respectively, than those obtained in this study. The kinetics of the formation and dissociation of this complex are not known precisely. It has been estimated[18] that the formation rate constant is about 10^8 M^{-1} s^{-1}. The formation constant has been reported[20] to be about 10^7 M^{-1}, and our own experiments support this (see the following section). The very large rate constant for formation of $Fe(II)(edta)(NO)^{2-}$ and its high stability makes it a good scavenger for NO [eqn. (4)].*

$$HrNO + Fe(II)(edta)^{2-} \rightleftharpoons Hr^{o} + Fe(II)(edta)(NO)^{2-} \quad (4)$$

The reaction was monitored at λ = 380, 410 or 510 nm using the stopped-flow instrument. The change of absorbance with time followed first-order kinetics for at

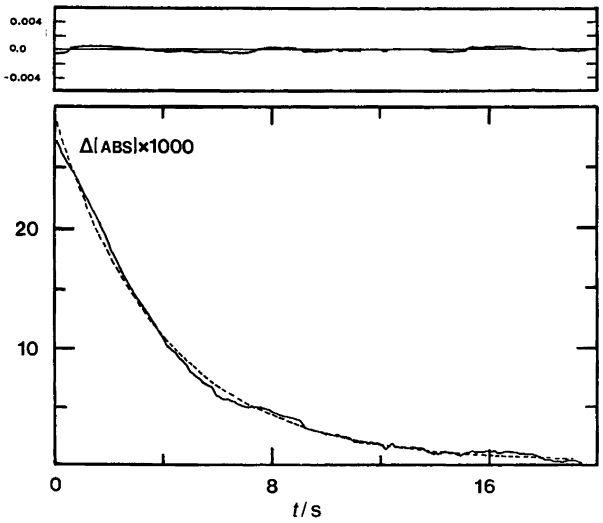

Fig. 6. Observed (——) and calculated (- - -) change of absorbance (410 nm) with time for the reaction of 1.3×10^{-5} M HrNO with 0.005 M $Fe(II)(edta)^{2-}$ in 0.1 M phosphate (pH 7) at 16.3 °C. The upper curve shows the residual ($ABS_{calc}-ABS_{obs}$).

* It is noted that for both $Fe(II)(edta)(NO)^{2-}$ and HrNO there is probably a substantial transfer of electron density from the metal to the nitrosyl group, leading to the limiting formulae $Fe(III)NO^{-}$ and $Fe(II),Fe(III)NO^{-}$, respectively (Ref. 14 and references therein).

Table 3. Kinetic data for the reaction of HrNO with various scavengers.[a]

10^5[HrNO][b] /M	[Scavenger] /M	k_{obs} /s^{-1}	k_d(calc)[c] /s^{-1}	T /°C
Scavenger: Fe(II)(edta)[d]				
1.3	0.01	2.8[e]	3.1	35.1
1.3	0.005	2.6[e]	3.1	35.1
1.3	0.002	3.3[e]	3.1	35.1
1.3	0.01	0.97[e,g]	0.84	25.0
4.4	0.005	0.90[e]	0.84	25.0
1.3	0.005	0.80[e]	0.84	25.0
1.3	0.002	0.86[e,f]	0.84	25.0
1.3	0.01	0.23[e]	0.26	16.3
1.3	0.005	0.24[e]	0.26	16.3
3.7[h]	0.005	0.29[e]	0.26	16.3
1.8[i]	0.005	0.28[e]	0.26	16.3
1.3	0.002	0.23[e]	0.26	16.3
Scavenger: oxygen				
1.9[j]	0.00055	0.91[k]	0.84	25.0
1.9[j]	0.00055	0.091[k]	0.103	10.0
1.8[j]	0.00055	0.043[k]	0.042	4.0
Scavenger: myoglobin				
2.0	0.00016	0.88[f]	0.84	25.0

[a]0.1 M potassium phosphate, pH 7 and $I = 0.2$ M. [b]Equimolar amounts of Hr° and NO if not stated otherwise. [c]Calculated from the activation energies in Table 2. [d]$[edta]_{total} = 2\times[Fe(II)]_{total}$. [e]Monitored at $\lambda = 410$ nm. Each value is the average of two separate experiments. [f]The same k_{obs} values were obtained when the reaction was monitored at $\lambda = 380$ and 510 nm. [g]At pH 8.1 using a 0.05 M Na_2SO_4, 0.05 M tris(hydroxymethyl) aminoethane buffer solution ($I = 0.2$ M) the value 1.0 s^{-1} was obtained. [h]$[NO]_{excess} = 1.4\times10^{-5}$ M. [i]$[Hr°]_{excess} = 1.0\times10^{-4}$ M. [k]Monitored at $\lambda = 500$ nm. [l]Monitored at $\lambda = 480$ and 560 nm.

least three half-lives, and there was no wavelength dependence (Fig. 6). As seen from Table 3 the same rate constants were obtained for $[HrNO] = 1.3\times10^{-5}$ M with $[Fe(II)(edta)^{2-}]$ varying from 0.01 to 0.002 M. There was also no variation for $[Fe(II)(edta)^{2-}] = 0.005$ M and $[HrNO] = (1.5\text{–}3.7)\times10^{-5}$ M. All the experiments listed in Table 3 were carried out using a phosphate buffer with pH 7.0, and additional measurements at pH 8.1 showed no dependence on pH within experimental error. Most of the experiments were performed using equimolar amounts of Hr° and NO. However, using a very large excess of Hr° or NO gave nearly the same rate constant (Table 3).

The changes in absorbance were proportional to the initial concentration of HrNO, but independent of an excess of either Hr° or NO, and also independent of the concentration of $Fe(II)(edta)^{2-}$. However, the observed changes in absorbance were 90±10 % of those calculated by assuming that the reaction between Hr° and the Fe(II)(edta)$^{2-}$ complex was quantitative, eqn. (4), using the known molar absorbances of the species involved. These deviations, which were unsystematic, were explained by contamination of the reactant solutions with traces of oxygen. The kinetic data are interpreted in terms of a slow dissociation of HrNO followed by a much faster reaction between $Fe(II)(edta)^{2-}$ and NO as shown in eqns. (5) and (6).

$$HrNO \underset{k_f}{\overset{k_d}{\rightleftharpoons}} Hr° + NO \tag{5}$$

$$Fe(II)(edta)^{2-} + NO \underset{k_{-1}}{\overset{\overset{K_1}{k_1}}{\rightleftharpoons}} Fe(II)(edta)(NO)^{2-} \tag{6}$$

The steady-state expression for this reaction sequence is shown in eqn. (7). It is seen that for $[Hr°] \ll [Fe(II)(edta)^{2-}]$

$$k_{obs} = \frac{k_d k_1 [Fe(II)(edta)^{2-}]}{k_f[Hr°] + k_1[Fe(II)(edta)^{2-}]} \tag{7}$$

we have $k_f[Hr°] \ll k_1[Fe(II)(edta)^{2-}]$, since $k_f < k_1$, and therefore the expression is reduced to eqn. (8).

$$k_{obs} = k_d \tag{8}$$

This predicts that k_{obs} should be independent of the concentrations of both Hr° and Fe(II)(edta), in agreement with the experimental results shown in Table 3.

Oxygen as scavenger. Oxygen is an effective scavenger, since it reacts with Hr°, forming HrO_2, and with NO, forming HONO. Thus, when oxygen in excess is added to a solution of HrNO a quantitative formation of HrO_2 occurs, as shown spectrophotometrically. The reaction between Hr° and oxygen is fast ($k = 7.4\times10^6$ M^{-1} s^{-1} at 25 °C), and from the formation constant ($K = 1.5\times10^5$ M^{-1}) it is seen that practically all the protein is bound to oxygen at moderate concentrations of oxygen.[5] The reaction between NO and O_2 has not been studied in detail, but in moderate concentrations it is very fast compared to the dissociation of HrNO (see below). Both products are therefore scavenged very effectively by oxygen if only the concentration of the scavenger is sufficiently large, in which case the rate constant, as with $Fe(II)(edta)^{2-}$ above, should be equal to k_d. The kinetics of the reaction were studied by reacting 0.02 mM HrNO with 0.55 mM oxygen. The reaction was monitored at 500 nm. The change in absorbance was equal (±10 %) to that calculated from the known molar absorbances and followed first-order kinetics for at least three half-lives. The values of k_{obs} determined at three different temperatures are listed in Table 3. The observed rate constants are in excellent agreement with those obtained above using $Fe(II)(edta)^{2-}$ as a scavenger, and thus support the interpretation that $k_{obs} = k_d$.

In aqueous solution nitric oxide is oxidized by oxygen,

forming nitrous acid or nitrite depending on the pH, as shown in eqn. (9) for pH 7.

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (9)$$

The reaction can (in principle) be monitored at 355 nm, where nitrite has an absorption maxima, while nitric oxide or oxygen does not absorb at this wavelength. However, our preliminary measurements showed that the reaction is very fast, and we only obtained an upper limit to the rate constant. Spectrophotometric measurements in the region $\lambda = 250$–400 nm and stopped-flow measurements at $\lambda = 355$ nm showed that for a solution initially containing 10^{-4} M NO and an excess of oxygen ($[O_2]_{av} = 0.9\times10^{-4}$ M) the reaction was >80 % complete within 6 ms at 25 °C. This experiment does not establish the reaction order, which has been reported to be third order[21] (rate $= k[NO]^2[O_2]$ with $k = 9\times10^6$ M^{-2} s^{-1}). However, our results indicate that the value 4×10^{10} M^{-2} s^{-1} can be taken as a lower-limit estimate for the third-order rate constant, in contrast to the reported value. Our results clearly demonstrate that the reaction between nitric oxide and oxygen is very fast and that under the experimental conditions used in the scavenging experiments is order of magnitude faster than the dissociation of nitric oxide from deoxyhemerythrin.

Myoglobin as scavenger. Sperm whale deoxymyoglobin reacts rapidly with NO ($k = 1.7\times10^7$ M^{-1} s^{-1} at pH 7, 0.05 M phosphate, 20 °C) forming a very stable adduct ($K = 1.5\times10^{11}$ M^{-1}).[22] The reaction of HrNO with an excess of myoglobin (monitored at $\lambda = 480$ and 560 nm) gave a k_{obs} value similar to those observed using the other scavengers (Table 3). This suggests that Mb° also acts as an efficient scavenger.

Activation parameters. From the temperature dependence of the rate constant for dissociation, k_d, the activation parameters were determined. This was carried out by least-squares calculations including all the values of k_d determined using the three scavengers discussed above (Table 2). Note that both parameters are well-defined and that for each of the scavengers there is good agreement between the observed and calculated values of k_d (Table 3, Fig. 5).

Thermodynamic parameters. The equilibrium constant $K_f = k_f/k_d$ and its $\Delta H^\ominus$ and $\Delta S^\ominus$ values were calculated from the kinetic data determined above (Table 2). It is noted that each of the thermodynamic parameters are well-defined.

Reaction of oxyhemerythrin with nitric oxide. The reaction of oxyhemerythrin with an excess of NO gave HrNO quantitatively shown spectrophotometrically. The reaction of 5×10^{-5} M HrO_2 with 5×10^{-4} M NO was monitored at 500 nm using stopped-flow methods. The change of absorbance followed first-order kinetics and gave $k_{obs} = 43$ s^{-1} at 25 °C. This value is similar to the reported value[5] for dissociation of HrO_2 (51 s^{-1}), which strongly suggests that NO under these circumstances acts as an effecient scavenger. This also indicates that the two gases occupy the same iron(II) site, as discussed further in the last section. We note that the present result is in contrast to a recent report that the reaction of oxymyoglobin with an excess of NO is very fast ($t_{1/2} = 2$ s) and affords a quantitative oxidation to metmyoglobin and nitrate.[23] A similar result was reported for hemoglobin. We obtained no indication that such a process takes place with the present system.

Formation constant of Fe(II)(edta)(NO)$^{2-}$. The equilibrium constant for the binding of NO to Fe(II)(edta)$^{2-}$ [eqn. (6)] was determined by studying the competition between Hr° and Fe(II)(edta)$^{2-}$ for a deficiency of NO. Thus, when Fe(II)(edta)$^{2-}$ is added to a solution of HrNO it was shown spectrophotometrically that all the nitric oxide becomes bound to Fe(II)(edta)$^{2-}$ if $C_{Fe(II)(edta)}/C_{Hr°} \geq 10$ [eqn. (4)]. At smaller $C_{Fe(II)(edta)}/C_{Hr°}$ ratios significant amounts of both Fe(II)(edta)(NO)$^{2-}$ and HrNO are present, and the ratio [Fe(II)(edta)(NO)$^{2-}$]/[HrNO] was determined spectrophotometrically ($\lambda = 500$, 550 and 600 nm; no dependence on wavelength was observed). For a mixture with $C_{Fe(II)(edta)} = 0.248$ mM, $C_{Hr°} = 0.566$ mM and $C_{NO} = 0.30$ mM the ratio [Fe(II)(edta)(NO)$^{2-}$]/[HrNO] was determined to be 0.77. The actual concentration of each of the species may then be calculated from the solution stoichiometry (free NO can be ignored), and the ratio K_f/K_1 [see eqn. (10)] may then be calculated. This gave $K_f/K_1 = 0.38$.

$$K_f/K_1 = [HrNO][Fe(II)(edta)^{2-}]/[Hr°][Fe(II)(edta)(NO)^{2-}] \quad (10)$$

A similar experiment with $C_{Fe(II)(edta)} = 0.475$ mM, $C_{Hr°} = 0.543$ mM and $C_{NO} = 0.288$ mM gave [Fe(II)(edta)(NO)$^{2-}$]/[HrNO] = 2.1, and this gave $K_f/K_1 = 0.30$. Using the average value $K_f/K_1 = 0.34$ combined with the value $K_f = 5.0\times10^6$ M determined above, the value $K_1 = 1.5\times10^7$ M was calculated (25 °C, 0.1 M potassium phosphate, pH 7). This value is in good agreement with the values previously determined by Hishinuma *et al.* on the basis of equilibrium studies.[20] Combining our determination at 25 °C with those

Table 4. The equilibrium constant for the formation of Fe(II)(edta)(NO)$^{2-}$.[a]

$10^{-7}K_{obs}$ /M^{-1}	$10^{-7}K_{calc}$[d] /M^{-1}	T /°C	Ref.
1.5[b]	1.45	25.0	This work
0.35[c]	0.37	38.5	20
0.086[c]	0.083	55.0	–
0.024[c]	0.024	70.0	–

[a]See eqn. (6). [b]0.1 M potassium phosphate, pH 7.0 and $I = 0.2$ M. [c]Water, [Fe(II)(edta)(NO)$^{2-}$] = 0.01–0.04 M, ca. pH 7. [d]Calculated from $\Delta H^\ominus = -77.5$ kJ mol^{-1} and $\Delta S^\ominus = -123$ J mol^{-1} K^{-1}.

from Ref. 20 we obtained the values $\Delta H^{\ominus} = -77.5 \pm 1.5$ kJ mol^{-1} and $\Delta S^{\ominus} = -123 \pm 5$ mol^{-1} K^{-1}. From Table 4 it is seen that these parameters give a good agreement between the calculated and observed equilibrium constants for the entire temperature region. The above values for $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ are significantly different from those reported in Ref. 20; however, calculations using only the data given in Ref. 20 do not give the parameters quoted in this reference, but result in nearly the same parameters as above, when our value for 25 °C is included.

Comparison with other systems. The rapid reaction of NO with deoxyhemerythrin to yield the NO adduct could only be studied by stopped-flow methods, and then only with some difficulty. The rate of dissociation of the adduct falls nicely in the stopped-flow range, and three quite different scavengers were effective. Relaxation methods used with HrO_2[6-8] were not succesful with the HrNO system. All kinetic and thermodynamic data are collected in Table 2 and are compared to those for O_2, HN_3, HCNO and HF binding.[5,12] The different conditions have little effect on the rate constants[5,12] and the comparison is valid. It appears from these and previous results that the five ligands bind at the same position (Fig. 1) and that ligand interchange only occurs by a dissociative mechanism (i.e. there is no ligand-assisted path). It is noted that a recent spectroscopic study of HrNO also indicates that NO is bound at the same position as O_2.[14]

As might be anticipated, the behaviour of NO resembles that of O_2 and both display distinct differences from the HX group. Both NO and O_2 have large $\Delta H^{\ddagger}$ values for the on- and (particularly) off-processes. Thus the interactions of NO and O_2 are much more exothermic than are those of HX. The interaction of Hr° with the two gases have more positive $\Delta S^{\ddagger}$values and more negative $\Delta S^{\ominus}$ values than with HX. The enhanced stability of the NO over the O_2 adduct resides almost solely in a smaller k_d and a less negative $\Delta S^{\ominus}$ of reaction.

The binding of NO to Hr°, as with that of O_2, shows no pH effect, and the rate constant is independent of the degree of NO complexing. Thus NO (and O_2) binding with *P. gouldii* octamer shows no cooperativity.

Comparison of the kinetic and thermodynamic data for NO adducts of hemerythrin, myoglobin[22] and some mononuclear iron(II) complexes [$Fe(H_2O)_6^{2+}$, Fe(citrate)$^-$ and $Fe(edta)^{2-}$][18,22] shows that the formation constants span a very large range from 5×10^2 M^{-1} [$Fe(H_2O)_6^{2+}$] to 10^{11} M^{-1} (myoglobin). The rates of formation vary only by a factor 100 (10^5–10^7 M^{-1} s^{-1}). The variation in the stability is therefore essentially due to a very large variation of the rate of dissociation: from 10^{-4} s^{-1} (myoglobin) to 10^3 s^{-1} [$Fe(H_2O)_6^{2+}$]. The rate of dissociation of HrNO is intermediate, being somewhat closer to the values for the simple mononuclear species than to that of myoglobin.

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