Biochemical and Biophysical Research Communications 393 (2010) 196–200

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reductive nitrosylation of ferric cyanide horse heart myoglobin is limited by cyanide dissociation

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ARTICLE INFO

Article history:
Received 13 January 2010
Available online 29 January 2010

This paper is dedicated to Beatrice A. Wittemberg who pioneered cyanide binding to globins.

Keywords:
Ferric cyanide horse heart myoglobin
Nitrogen monoxide
Reductive nitrosylation
Kinetics

ABSTRACT

Cyanide binds to ferric heme-proteins with a very high affinity, reflecting the very low dissociation rate constant (k_{off}). Since no techniques are available to estimate k_{off}, we report herewith a method to determine k_{off} based on the irreversible reductive nitrosylation reaction to trap ferric myoglobin (Mb(III)). The k_{off} value for cyanide dissociation from ferric cyanide horse heart myoglobin (Mb(III)–cyanide) was determined at pH 9.2 and 20.0 °C. Mixing Mb(III)–cyanide and NO solutions brings about absorption spectral changes reflecting the disappearance of Mb(III)–cyanide with the concomitant formation of ferrous nitrosylated Mb. Since kinetics of reductive nitrosylation of Mb(III) is much faster than Mb(III)–cyanide dissociation, the k_{off} value, representing the rate-limiting step, can be directly determined. The k_{off} value obtained experimentally matches very well to that calculated from values of the second-order rate constant (k_{on}) and of the dissociation equilibrium constant (K) for cyanide binding to Mb(III) ($k_{off} = k_{on} \times K$).

Cyanide is one of the few ligands able to interact with both ferric and ferrous heme-proteins, albeit with very different thermodynamic and kinetic parameters. Cyanide binds to ferric heme-proteins with a very high affinity; values of the dissociation equilibrium constant (*i.e.*, K) have been estimated to be lower than 10^{-5} M. This reflects primarily the very low first-order rate constant of cyanide dissociation (*i.e.*, k_{off}) ranging between 10^{-2} s^{-1} and 10^{-7} s^{-1}. In fact, values of the second-order rate constant for cyanide binding to ferric heme-proteins (*i.e.*, k_{on}) range between 10^{2} M^{-1} s^{-1} and 10^{4} M^{-1} s^{-1}. In contrast, the affinity of cyanide for ferrous heme-proteins is low, values of the dissociation equilibrium constant (*i.e.*, D) being usually higher than 10^{-2} M. In fact, values of the first-order rate constant for cyanide dissociation from ferrous heme-proteins (*i.e.*, d_{off}) range between 10^{-2} s^{-1} and 1 s^{-1}, and values of the second-order rate constant for cyanide binding to ferrous heme-proteins (*i.e.*, d_{on}) vary between 5×10^{-1} M^{-1} s^{-1} and 5 M^{-1} s^{-1}. Only ferrous *Campylobacter jejuni* truncated-hemoglobin P displays a very high reactivity for cyanide, reflecting an unusual stabilization mode of the heme-bound cyanide. Indeed, the X-ray crystal structure of the cyanide derivative shows that the ligand is hydrogen bonded to the phenolic OH group of TyrB10 and to the indole nitrogen atom of TrpG8 [1,2].

Although the reaction of cyanide with ferric heme-proteins has received large attention, only values of k_{on} are usually determined due to the very high ligand affinity and the very slow dissociation kinetics [1,2]. It is important to outline that a puzzling unexplained feature of the equilibrium curves of ferric horse heart myoglobin (Mb(III)) with cyanide is the value of the Hill coefficient n, which is considerably higher than 1, that is a paradoxical result for a monomeric heme-protein. However, this feature might be related to either (*i*) the very slow approach to equilibrium at low cyanide concentration, and/or (*ii*) the uncertainty on the determination of free cyanide at low cyanide concentration [3]. Furthermore, no methods are available to determine directly k_{off}, which is estimated generally from values of k_{on} and K (*i.e.*, $k_{off} = k_{on} \times K$).

The present study reports a method, based on the irreversible reductive nitrosylation reaction to trap cyanide-free Mb(III), which allows to determine directly the k_{off} value for cyanide dissociation from ferric cyanide horse heart myoglobin (Mb(III)–cyanide). Mixing of Mb(III)–cyanide and NO solutions induces the disappearance of Mb(III)–cyanide with the concomitant formation of ferrous nitrosylated Mb (Mb(II)–NO). Since Mb(III)–cyanide dissociation represents the rate-limiting step of the whole process, the k_{off} value

Abbreviations: heme-Fe(III), ferric heme-protein; heme-Fe(III)-cyanide, ferric cyanide heme-protein; heme-Fe(II), ferrous heme-protein; Mb, myoglobin; Mb(III), ferric Mb; Mb(III)–cyanide, ferric cyanide Mb; Mb(III)–NO, ferric nitrosylated Mb; Mb(II), ferrous Mb; Mb(II)–NO, ferrous nitrosylated Mb.

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doi:10.1016/j.bbrc.2010.01.092

can be easily determined. Moreover, both K and k_{on} values for cyanide binding to Mb(III) have been determined. As expected for a simple system [3], the k_{off} value obtained experimentally matches very well to that calculated from values of k_{on} and K (*i.e.*, $k_{off} = k_{on} \times K$) and the equilibrium curve is characterized by a Hill coefficient $n = 1.01 \pm 0.02$.

Materials and methods

Horse heart Mb(III) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The Mb(III) solution (5.6×10^{-5} M) was prepared by dissolving Mb(III) in 2% borate buffer, pH 9.2, at 20.0 °C. The Mb(III) concentration was determined spectrophometrically with values of λ_{max} and ε given in Table 1S [3].

NO (from Aldrich Chemical Co., Milwaukee, WI, USA) was purified by flowing through an NaOH column in order to remove acidic nitrogen oxides. The NO stock solution was prepared by keeping in a closed vessel the 2.0% borate buffer solution (pH 9.2) under NO at $P = 760.0$ mm Hg anaerobically ($T = 20.0$ °C). The solubility of NO in the aqueous buffered solution is 2.05×10^{-3} M, at $P = 760.0$ mm Hg and $T = 20.0$ °C [3]. All the other products (from Merck AG, Darmstadt, Germany; and Sigma–Aldrich, St. Louis, MO, USA) were of analytical grade and used without purification unless stated.

The horse heart Mb(III)–cyanide solution (5.6×10^{-6} M and 5.6×10^{-5} M) was obtained by adding a 10-molar excess of the cyanide stock solution (1.0×10^{-2} M) to the Mb(III) solution (5.6×10^{-6} M and 5.6×10^{-5} M) [3].

The horse heart Mb(II)–NO solution (5.6×10^{-6} M and 5.2×10^{-5} M) was obtained by adding a 10-molar excess of the NO stock solution (2.05×10^{-3} M) to the ferrous Mb (Mb(II)) solution (5.6×10^{-6} M and 5.6×10^{-5} M), in the presence of sodium dithionite (1.0×10^{-2} M) [3].

Kinetics and thermodynamics of reductive nitrosylation of cyanide-free and cyanide-bound horse heart Mb(III) and of cyanide binding to horse heart Mb(III) were analyzed in the framework of the minimum reaction mechanism represented by Scheme 1 [3–9].

$$\text{Mb(III)-cyanide} \underset{k_{on}}{\overset{k_{off}}{\rightleftarrows}} \text{Mb(III)} + \text{cyanide} \quad \text{(a)}$$

$$\text{Mb(III)} + \text{NO} \underset{h_{off}}{\overset{h_{on}}{\rightleftarrows}} \text{Mb(III)-NO} \quad \text{(b)}$$

$$\text{Mb(III)- NO} \overset{\text{fast}}{\rightleftarrows} \text{Mb(II)-NO}^{+} \quad \text{(c)}$$

$$\text{Mb(II)-NO}^{+} + \text{OH}^{-} \overset{l}{\rightarrow} \text{Mb(II)} + \text{HNO}_2 \quad \text{(d)}$$

$$\text{Mb(II)} + \text{NO} \overset{b_{on}}{\rightarrow} \text{Mb(II)-NO} \quad \text{(e)}$$

In the absence of cyanide, values of the (pseudo-)first-order rate constants h and l for horse heart Mb(III) reductive nitrosylation (*i.e.*, for NO binding to Mb(III) and for the OH^--mediated conversion of Mb(II)–NO^+ to Mb(II); reactions b and d in Scheme 1, respectively) were determined by mixing the Mb(III) (final concentration, 2.8×10^{-6} M and 2.8×10^{-5} M) solution with the NO (final concentration, 1.0×10^{-4} M–1.0×10^{-3} M) solution under anaerobic conditions, at pH 9.2 (2.0% borate buffer) and 20.0 °C; no gaseous phase was present [4–9].

Values of h and l were obtained according to Eqs. (1)–(3) [4–10]:

$$[\text{Mb(III)}]_t = [\text{Mb(III)}]_i \times e^{-h \times t} \quad (1)$$

$$[\text{Mb(III)-NO}]_t = [\text{Mb(III)}]_i \times (h \times ((e^{-h \times t}/(l-h)) + (e^{-l \times t}/(h-l)))) \quad (2)$$

$$[\text{Mb(II)-NO}]_t = [\text{Mb(III)}]_i - [\text{Mb(III)}]_t + [\text{Mb(III)-NO}]_t \quad (3)$$

Values of h_{on} and h_{off} (reaction a in Scheme 1) were determined from the dependence of h on the NO concentration (*i.e.*, [NO]), according to Eq. (4) [3]:

$$h = h_{on} \times [\text{NO}] + h_{off} \quad (4)$$

The value of the equilibrium dissociation constant H for horse heart Mb(III) reductive nitrosylation (*i.e.*, for NO binding to Mb(III)) was determined from the dependence of the molar fraction α of NO-bound Mb(III) on the free NO concentration (*i.e.*, [NO]), according to Eq. (5) [3]:

$$\alpha = [\text{NO}]/([\text{NO}] + H) \quad (5)$$

In the presence of cyanide, values of the first-order rate constant k_{off} for reductive nitrosylation of horse heart Mb(III) (*i.e.*, for cyanide dissociation from Mb(III)–cyanide complexes) were determined by mixing the Mb(III)–cyanide (final concentration, 2.8×10^{-6} M and 2.8×10^{-5} M) solution with the NO (final concentration, 1.0×10^{-4} M–1.0×10^{-3} M) solution under anaerobic conditions, at pH 9.2 (2.0% borate buffer) and 20.0 °C; no gaseous phase was present.

The k_{off} value was determined from data analysis, according to Eq. (6) [3]:

$$[\text{Mb(III)-cyanide}]_t = [\text{Mb(III)-cyanide}]_i \times e^{-k_{off} \times t} \quad (6)$$

Values of the pseudo-first-order rate constant k for cyanide binding to horse heart Mb(III) were obtained by mixing the Mb(III) (final concentration, 2.8×10^{-6} M and 2.8×10^{-5} M) solution with the cyanide (final concentration, 5.0×10^{-4} M–1.0×10^{-2} M) solution, at pH 9.2 (2.0% borate buffer) and 20.0 °C [3].

Values of k were determined from data analysis, according to Eq. (7) [3]:

$$[\text{Mb(III)}]_t = [\text{Mb(III)}]_i \times e^{-k \times t} \quad (7)$$

Values of the second-order rate constant k_{on} for cyanide binding to horse heart Mb(III) were determined from the dependence of k on the cyanide concentration (*i.e.*, [cyanide]), according to Eq. (8) [3]:

$$k = k_{on} \times [\text{cyanide}] \quad (8)$$

Kinetics was monitored spectrophotometrically between 380 nm and 460 nm and between 500 nm and 700 nm.

The value of the dissociation equilibrium constant K for cyanide binding to horse heart Mb(III) was obtained by mixing the Mb(III) solution (final concentration, 2.8×10^{-6} M) with the cyanide solution (final concentration, 5.0×10^{-7} M–1.0×10^{-5} M), at pH 9.2 (2.0% borate buffer) and 20.0 °C [3]. The equilibration time was 24 h.

The value of K was determined from the dependence of the molar fraction Y of cyanide-bound Mb(III) on the free cyanide concentration (*i.e.*, [cyanide]), according to Eq. (9) [3]:

$$Y = [\text{cyanide}]/([\text{cyanide}] + K) \quad (9)$$

Thermodynamics was monitored spectrophotometrically between 380 nm and 460 nm.

Values of the pseudo-first-order rate constant b for NO binding to horse heart Mb(II) were obtained by mixing the Mb(II) (final concentration, 1.4×10^{-6} M) solution with the NO (final concentration, 6.0×10^{-6} M–2.0×10^{-5} M) solution, at pH 9.2 (2.0% borate buffer) and 20.0 °C [11].

Values of b were determined from data analysis, according to Eq. (10) [11]:

$$[\text{Mb(II)}]_t = [\text{Mb(II)}]_i \times e^{-b \times t} \quad (10)$$

Values of the second-order rate constant b_{on} for NO binding to horse heart Mb(II) were determined from the dependence of b on the NO concentration (*i.e.*, [NO]), according to Eq. (11) [3]:

$$b = b_{on} \times [NO] \quad (11)$$

Kinetics was monitored spectrophotometrically between 380 nm and 460 nm.

The results are given as mean values of at least four experiments plus or minus the corresponding standard deviation. All data were analyzed using the Matlab program (The Math Works Inc., Natick, MA, USA).

Results and discussion

Mixing of the horse heart Mb(III) and NO solutions brings about a shift of the maximum of the absorption peaks from 411 nm (ε = 119 mM^{-1} cm^{-1}), 539 nm (ε = 8.7 mM^{-1} cm^{-1}), and 585 nm (ε = 7.6 mM^{-1} cm^{-1}) (*i.e.*, Mb(III)), to 418 nm (ε = 104 mM^{-1} cm^{-1}), 535 nm (ε = 12.8 mM^{-1} cm^{-1}), and 573 nm (ε = 11.5 mM^{-1} cm^{-1}) (*i.e.*, Mb(III)–NO) (Fig. 1 and Table 1S). Then, the Mb(III)–NO solution undergoes a shift of the maximum of the absorption peaks from 418 nm (ε = 104 mM^{-1} cm^{-1}), 535 nm (ε = 12.8 mM^{-1} cm^{-1}), and 573 nm (ε = 11.5 mM^{-1} cm^{-1}) (*i.e.*, Mb(III)–NO), to 417 nm (ε = 131 mM^{-1} cm^{-1}), 544 nm (ε = 11.5 mM^{-1} cm^{-1}), and 575 nm (ε = 10.6 mM^{-1} cm^{-1}) (*i.e.*, Mb(II)–NO) (Fig. 1 and Table 1S). Mb(II) was never detected spectrophotometrically because of its very fast reaction with NO (b_{on} = 1.6 × 10^7 M^{-1} s^{-1}) (Table 1 and Fig. 1S). Note that cyanide binding to heme-Fe(II)-proteins is negligible in comparison with heme-Fe(II) nitrosylation [2,12].

Over the whole NO concentration range explored, the time course for horse heart Mb(III) reductive nitrosylation corresponds to a biphasic process (Fig. 1); values of h and l are wavelength-independent at fixed NO concentration. The first step of kinetics for Mb(III) reductive nitrosylation (indicated by h_{on} in Scheme 1) is a bimolecular process as observed under pseudo-first order conditions (Fig. 1). The plot of h versus [NO] is linear (Eq. (4)), the slope corresponds to h_{on} (= 6.8 × 10^4 M^{-1} s^{-1}) (Table 1). The y intercept of plots of h versus [NO] corresponds to h_{off} (= 5.2 s^{-1}) (Table 1). In contrast, the second step (indicated by l in Scheme 1) follows a [NO]-independent mono-molecular behavior (Fig. 1); the average value of l Mb(II) formation is 6.2 × 10^{-3} s^{-1} (Table 1).

Fig. 1 shows the dependence of the molar fraction of Mb(III)–NO (*i.e.*, α) on the free NO concentration (*i.e.*, [NO]). The analysis of data according to Eq. (5) allowed to determine the value of H for NO binding to Mb(III) (= 1.2 × 10^{-4} M) (Table 1). Consistently with the stoichiometry of reaction b in Scheme 1, the value of the Hill coefficient n is 1.00 ± 0.02. As expected for simple systems [3], the value of H (= h_{off}/h_{on}) determined experimentally (= 1.2 × 10^{-4} M) is in agreement with that of the h_{off}/h_{on} ratio (= 7.6 × 10^{-5} M) (Table 1).

Table 1
Kinetic and thermodynamic parameters for reductive nitrosylation of horse heart Mb(III) and Mb(III)–cyanide and for cyanide binding to horse heart Mb(III), at pH 9.2 and 20.0 °C. [a].

Reductive nitrosylation	
Mb(III)	h_{on} = 6.8 × 10^4 M^{-1} s^{-1} h_{off} = 5.2 s^{-1} H = 1.2 × 10^{-4} M $H = h_{off}/h_{on}$ = 7.6 × 10^{-5} M l = 6.2 × 10^{-3} s^{-1} b_{on} = 1.6 × 10^7 M^{-1} s^{-1}
Mb(III)–cyanide	k_{off} = 4.3 × 10^{-4} s^{-1}
Cyanide binding	
Mb(III)	k_{on} = 3.1 × 10^2 M^{-1} s^{-1} K = 1.3 × 10^{-6} M $k_{off} = k_{on} \times K$ = 4.4 × 10^{-4} s^{-1}

[a] For details, see Scheme 1.

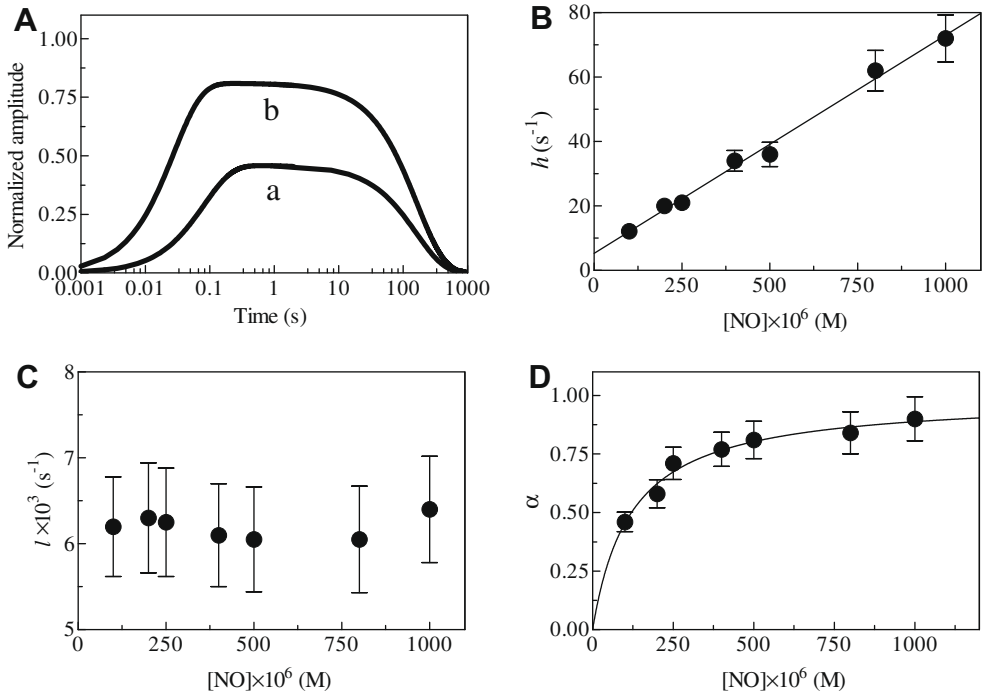

Fig. 1. Reductive nitrosylation of horse heart Mb(III), at pH 9.2 and 20.0 °C. (A) Normalized averaged time courses of Mb(III) reductive nitrosylation. The NO concentration was 1.0 × 10^{-4} M (trace a) and 5.0 × 10^{-4} M (trace b). The time course analysis according to Eq. (1–3) allowed the determination of the following values of h, l, and Y: trace a – h = 1.2 × 10^1 s^{-1}, l = 6.2 × 10^{-3} s^{-1}, and α = 0.46; and trace b – h = 3.6 × 10^1 s^{-1}, l = 6.1 × 10^{-3} s^{-1}, and α = 0.81. (B) Dependence of h for Mb(III) reductive nitrosylation on [NO]. The continuous line was generated from Eq. (2) with h_{on} = (6.8 ± 0.4)×10^4 M^{-1} s^{-1} and h_{off} = 5.2 ± 2.0 s^{-1}. (C) Dependence of l for Mb(III) reductive nitrosylation on [NO]. The average l value is l = (6.2 ± 0.2) × 10^{-3} s^{-1}. (D) Dependence of α for Mb(III) reductive nitrosylation on free [NO]. The continuous line was generated from Eq. (5) with H = (1.2 ± 0.3)×10^{-4} M. The Mb(III) concentration was 2.8 × 10^{-6} M and 2.8 × 10^{-5} M. For details, see text.

Mixing of horse heart Mb(III)–cyanide and NO solutions induces a shift of the maximum of the absorption peaks from 422 nm ($\varepsilon = 116\ mM^{-1}\ cm^{-1}$) and 541 nm ($\varepsilon = 11.2\ mM^{-1}\ cm^{-1}$) (*i.e.*, Mb(III)–cyanide) to 417 nm ($\varepsilon = 131\ mM^{-1}\ cm^{-1}$), 544 nm ($\varepsilon = 11.5\ mM^{-1}\ cm^{-1}$), and 575 nm ($\varepsilon = 10.6\ mM^{-1}\ cm^{-1}$) (*i.e.*, Mb(II)–NO) (Fig. 2 and Table 1S).

Over the whole NO concentration range explored, the time course of Mb(III)–cyanide reductive nitrosylation corresponds to a mono-exponential process for more than 95% of its course (Fig. 2). The value of the first-order rate constant for reductive nitrosylation of Mb(III)–cyanide is wavelength- and [NO]-independent (Fig. 2), the average value being $4.3 \times 10^{-4}\ s^{-1}$ (Table 1).

Remarkably, (*i*) the Mb(III), Mb(III)–NO, and Mb(II) species were never detected spectrophotometrically, (*ii*) the value of the first-order rate constant for reductive nitrosylation of Mb(III)–cyanide (= $4.3 \times 10^{-4}\ s^{-1}$) is lower than those of kinetic parameters for reductive nitrosylation of Mb(III) by several orders of magnitude (Table 1), and (*iii*) the value of the first-order rate constant for reductive nitrosylation of Mb(III)–cyanide is reminiscent to those reported in the literature for cyanide dissociation from heme-Fe(III)–cyanide complexes [1,2]. These data suggest that cyanide dissociation from horse heart Mb(III)–cyanide represents the rate-limiting step of reductive nitrosylation, as described in Scheme 1. Therefore, the first-order rate constant for reductive nitrosylation of Mb(III)–cyanide corresponds to the first-order rate constant for cyanide dissociation, *i.e.*, k_{off} (reaction *a* in Scheme 1).

Mixing of the horse heart Mb(III) and cyanide solutions brings about a shift of the maximum of the absorption peaks from 411 nm ($\varepsilon = 119\ mM^{-1}\ cm^{-1}$), 539 nm ($\varepsilon = 8.7\ mM^{-1}\ cm^{-1}$), and 585 nm ($\varepsilon = 7.6\ mM^{-1}\ cm^{-1}$) (*i.e.*, Mb(III)) to 422 nm ($\varepsilon = 116\ mM^{-1}\ cm^{-1}$) and 541 nm ($11.2\ mM^{-1}\ cm^{-1}$) (*i.e.*, Mb(III)–cyanide) (Table 1S).

The time course of cyanide binding to horse heart Mb(III) corresponds to a mono-exponential process for more than 95% of its course. Values of the pseudo-first-order rate constant for Mb(III)–cyanide formation (*i.e.*, *k*) are wavelength-independent at fixed cyanide concentration. The plot of *k* versus [cyanide] is linear (Eq. (8)), the slope of the plot corresponds to k_{on} (Fig. 3). The value of k_{on} for cyanide binding to horse heart Mb(III) (= $3.4 \times 10^{2}\ M^{-1}\ s^{-1}$) (Table 1) is closely similar to that reported in the literature (= $3.1 \times 10^{2}\ M^{-1}\ s^{-1}$, at pH 9.1 and 21–23 °C) [3].

Fig. 3 shows the dependence of the molar fraction of Mb(III)–cyanide (*i.e.*, *Y*) on the free cyanide concentration (*i.e.*, [cyanide]). The analysis of data according to Eq. (9) allowed to determine the value of *K* for cyanide binding to Mb(III) (= 1.3×10^{-6} M) (Table 1). Consistently with the stoichiometry of reaction *a* in Scheme 1, the value of the Hill coefficient *n* is 1.01 ± 0.02. The value of *K* here determined (Table 1) agrees with that reported in the literature (= 4.4×10^{-6} M, at pH 9.1 and 21–23 °C) [3].

A very important point to underline is that this represents the first direct measurement of the cyanide dissociation kinetics from horse heart Mb(III)–cyanide, which exploits the fact that the reductive nitrosylation of horse heart Mb(III)–cyanide occurs with a reaction mechanism in which cyanide dissociation represents the rate-limiting step. Therefore, heme-Fe(III) reduction by NO takes place with unliganded Mb(III) only after the cyanide dissociation.

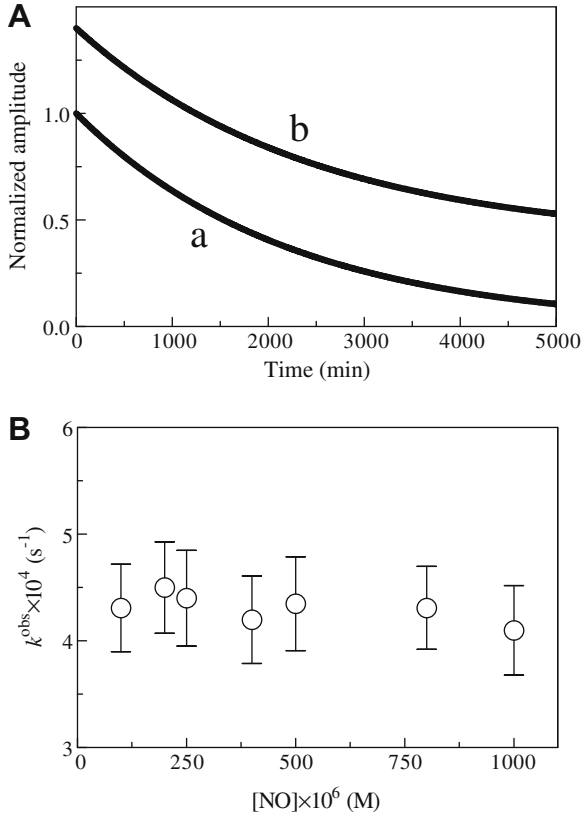

Fig. 2. Reductive nitrosylation of cyanide-bound horse heart Mb(III), at pH 9.2 and 20.0 °C. (A) Normalized averaged time courses for reductive nitrosylation of Mb(III)–cyanide. The NO concentration was 2.0×10^{-4} M (trace a) and 1.0×10^{-3} M (trace b). The time course analysis according to Eq. (6) allowed the determination of the following values of $k_{off} = 4.5 \times 10^{-4}\ s^{-1}$ (trace a) and $4.1 \times 10^{-4}\ s^{-1}$ (trace b). (B) Dependence of k_{off} for reductive nitrosylation of Mb(III)–cyanide on [NO]. The average k_{off} value is $4.3 \times 10^{-4}\ s^{-1}$. The Mb(III) concentration was 2.8×10^{-6} M and 2.8×10^{-5} M. For details, see text.

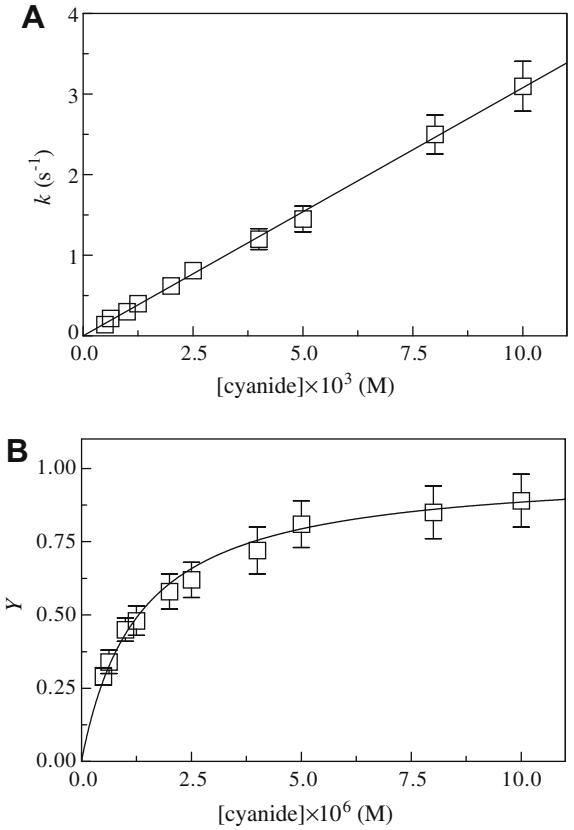

Fig. 3. Kinetics and thermodynamics of cyanide binding to horse heart Mb(III), at pH 9.2 and 20.0 °C. (A) Dependence of *k* for cyanide binding to Mb(III) on [cyanide]. The continuous line was generated from Eq. (8) with $k_{on} = (3.1 \pm 0.3) \times 10^{2}\ M^{-1}\ s^{-1}$. The Mb(III) concentration was 2.8×10^{-6} M. (B) Dependence of *Y* for cyanide binding to Mb(III) on the free ligand concentration (*i.e.*, [cyanide]). The continuous line was generated from Eq. (9) with $K = (1.3 \pm 0.2) \times 10^{-6}$ M. The Mb(III) concentration was 1.4×10^{-6} M. For details, see text.

To our knowledge, the use of NO is the only way to observe directly the cyanide dissociation process from Mb(III)–cyanide, since for this observation to be correct we need that the cyanide dissociation process is the only rate-limiting step. Therefore, a caution must be applied in checking that, indeed this is true under the experimental conditions employed.

As a matter of fact, the measurements reported in this work have been carried at pH > 9.0, because at lower pH values the step (*d*) of Scheme 1 becomes slow enough to affect the observed measurements [4,5]. On the other hand, caution must be also paid on that the observed cyanide dissociation process occurs for the Mb(III)–cyanide complex. In this respect, this mechanism is completely different from that occurring upon Mb(III)–cyanide reduction by sodium dithionite, which involves instead the transient formation of the heme-Fe(II)–cyanide species, which then decays to heme-Fe(II) due to its very low affinity for cyanide [1,2,13]. Thus, in this last case only cyanide dissociation from Mb(II)–cyanide can be investigated. As a whole, data here reported represent the first quantitative analysis of reductive nitrosylation (*i.e.*, NO-mediated reduction) of the cyanide derivative of a heme-Fe(III)-protein which appears to be limited by cyanide dissociation kinetics. This study opens the possibility to determine quantitatively cyanide dissociation kinetics from heme-Fe(III)-proteins. The correctness of the approach presented in this work is strengthened by the fact that the k_{off} value obtained experimentally (= 4.3×10^{-4} s^{-1}) matches well to that calculated from values of k_{on} and K for cyanide binding to horse heart Mb(III) ($k_{off} = k_{on} \times K = 4.4 \times 10^{-4}$ s^{-1}) (Table 1).

In conclusion, the different mechanisms describing NO- and dithionite-induced reduction of horse heart Mb(III)–cyanide (see present study and [1,2]) are important since reduction of Mb(III)–cyanide may be used to determine the first-order rate constant of cyanide dissociation (*i.e.*, k_{off}). However, some caution is demanded since cyanide dissociation must be slower than heme-Fe(III)-atom reduction, as reported here for reductive nitrosylation of Mb(III)–cyanide.

Acknowledgements

This work was partially supported by grants from the Ministry for University and Research of Italy (University 'Roma Tre', Roma, Italy, 'CLAR 2009' to P.A. and University of Roma 'Tor Vergata', Roma, Italy, '60%' to M.C.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.092.

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