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

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

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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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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]:

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