Biomimetic studies related to the azide-inhibited Cu,Zn superoxide dismutases

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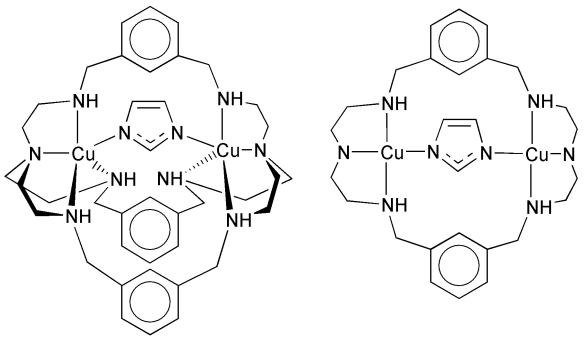
Azide binding to imidazolate-bridged dinuclear copper(II) complexes of macrocyclic (L′) and macrobicyclic (L) ligands has been investigated by EPR, UV-visible spectrophotometry and kinetics. The EPR spectra show that an azido bridge replaces the imidazolate bridge in the macrobicyclic complex while the azide anion is bound to one copper and the imidazolate bridge is broken in the macrocyclic complex. The rate constant of azide binding is very low for the complexes without labile coordination and relatively large for those with labile coordination, whatever the macrocycle or macrobicycle structure of the ligand.

Copper, zinc superoxide dismutases (Cu,Zn SODs) contain in their active site an imidazolate-bridged bimetallic center with one copper(II) and one zinc(II) ion.[1] They are ubiquitous metalloproteins found in eukaryotic cells and they are believed to protect cells from the toxic effects of superoxide ions by catalyzing the dismutation reaction into oxygen and hydrogen peroxide by alternating reduction and oxidation of the Cu(II) ion, which constitutes the active redox center.[2] The enzyme from different sources has been well characterized through crystallographic analysis[1,3–6] and other physicochemical studies.[7,8] The interaction of SOD with anions has been widely studied, since singly charged anions act as inhibitors of the enzyme activity.[9,10] The crystal structure of the azide-inhibited bovine Cu,Zn SOD has been described,[11] evidencing the direct coordination of the azide anion to the Cu(II) in place of the metal-bound water molecule, thus mimicking the enzyme–substrate interaction. The crystal structure of the azide adduct (at pH 5) of the Cu(I)–Zn reduced form of SOD has also been described.[12] The direct binding of the azide ion has also been evidenced from EPR and electronic studies.[13]

We have described two biomimetic models for the structure and the spectroscopic properties of the Cu,Cu-modified SOD $\{[\{Cu(im)Cu\}L]^{3+}$, Scheme 1$\}$ and Cu,Zn SOD $\{[\{Cu(im)Zn\}L]^{3+}\}$ respectively.[14] The two models effectively catalyze the dismutation of superoxide anion, even in the presence of bovine serum albumin (BSA), a strong biological copper(II) ion complexing agent. These models, which use a cryptand-type ligand L, revealed a large pH stability range.



$[(Cu(im)Cu)L]^{3+}$ $[(Cu(im)Cu)L']^{3+}$

Scheme 1 Models for SOD (the three perchlorate counter anions have been omitted for clarity).

We have also described the imidazolate-bridged dicopper complex of a macrocyclic ligand L′ $\{[\{Cu(im)Cu\}L']^{3+}$, Scheme 1$\}$ and evidenced the effect of the macrobicycle on the physicochemical properties, the stability *vs.* pH and the catalytic activity.[15] In this paper, we report solution studies of the reaction of azide ions with the two imidazolate-bridged dicopper complexes. For comparison, we also describe the reaction of azide ions with dicopper complexes in which the imidazolate bridge is lacking. This study is the first example of a biomimetic approach to the inhibition of SOD by azide ions.

Results

EPR studies

The EPR spectral changes produced by addition of azide to the complexes were followed by recording spectra in frozen water–DMSO (1 : 1 v/v) solutions. The spectra of the complex $[\{Cu(im)Cu\}L]^{3+}$ and of its azide adduct recorded at 100 K are shown in Fig. 1. The spectrum of $[\{Cu(im)Cu\}L]^{3+}$ (trace a) exhibits the features of an antiferromagnetically coupled dinuclear species.[14] The $\Delta M_s = 2$ transition is observed with seven clearly resolved copper hyperfine lines around 150 mT and the $\Delta M_s = 1$ region shows two broad signals at 290 and 360 mT assigned to the zero-field splitting interaction. The spectrum of the complex after addition of a large excess of azide (trace b) exhibits new signals showing the typical pattern of an azido-bridged dicopper cryptate $[\{Cu(N_3)Cu\}L]^{3+}$ by comparison with an authentic sample of $[\{Cu(N_3)Cu\}L]^{3+}$. Indeed, the spectrum of $[\{Cu(N_3)Cu\}L]^{3+}$ (trace c) shows a broad signal between 270 and 350 mT for the $\Delta M_s = 1$ region and another broad signal centered at 130 mT for the $\Delta M_s = 2$ region. This spectrum has already been published,[16] though not really interpreted, but magnetic susceptibility measurements[16] on $[\{Cu(N_3)Cu\}L]^{3+}$ show the presence of a weak ferromagnetic interaction: $-2J = -15$ cm^{-1} (ground state $S = 1$) and thus the complexity of the spectrum results from the many possibilities for transitions. Spectrum b shows that the solution contains $[\{Cu(N_3)Cu\}L]^{3+}$ and a small amount of the initial complex $[\{Cu(im)Cu\}L]^{3+}$. The EPR spectrum of a solution containing $[(Cu_2)L]^{4+}$ and an excess of azide (trace d) is similar to that of trace c, indicating the formation of the azido bridge between the two copper ions.

The EPR spectrum of the complex $[\{Cu(im)Cu\}L']^{3+}$ (Fig. 2, trace a) exhibits the expected spectral features of an anti-

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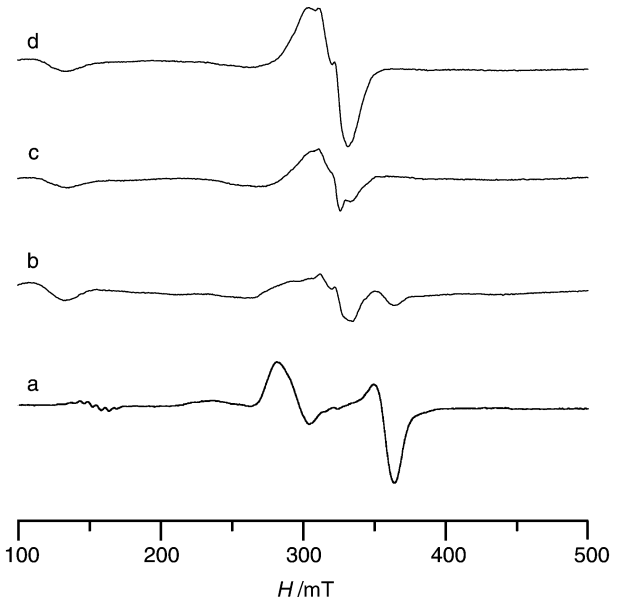


Fig. 1 EPR spectra at 100 K in water–DMSO (1 : 1 v/v): (a) $[\{Cu(im)Cu\}L]^{3+}$ (10^{-3} M), (b) $[\{Cu(im)Cu\}L]^{3+}$ (10^{-3} M) + N_3^- (0.25 M), (c) $[\{Cu(N_3)Cu\}L]^{3+}$ (10^{-3} M), (d) $[(Cu_2)L]^{4+}$ (10^{-3} M) + N_3^- (0.25 M).

ferromagnetically coupled dinuclear species with the $\Delta M_s = 1$ region showing two broad signals at 290 and 360 mT[15] and the $\Delta M_s = 2$ transition around 150 mT. The spectrum recorded after addition of an excess of azide to a solution of $[\{Cu(im)Cu\}L']^{3+}$ (trace c) shows the complete disappearance of the broad signals between 290 and 360 mT, revealing the formation of a complex with two different paramagnetic copper centers and no antiferromagnetically coupled dinuclear species. The EPR parameters for the azide adduct of $[\{Cu(im)Cu\}L']^{3+}$ were calculated from the simulation of the spectrum using the Brüker SIMFONIA program[17] and are listed in Table 1. Copper 1 and 2, corresponding to the two paramagnetic copper centers, do not have the same environment, since they present different EPR features. The ratios $g_{\|}/A_{\|}$ are an empirical index of tetrahedral distortion from a tetragonal geometry.[18] The values of 138 and 134 cm are indicative of square planar structures. All attempts to isolate a

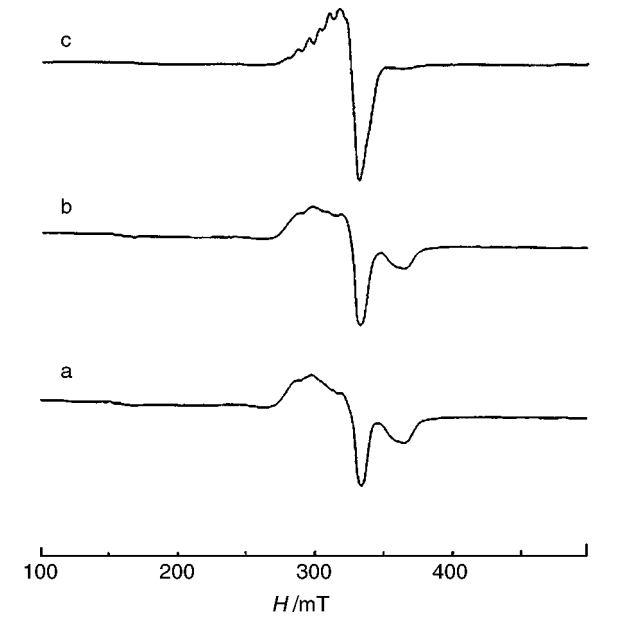


Fig. 2 EPR spectra at 100 K in water–DMSO (1 : 1 v/v): (a) $[\{Cu(im)Cu\}L']^{3+}$ (10^{-3} M), (b) $[\{Cu(im)Cu\}L']^{3+}$ (10^{-3} M) + N_3^- (10^{-3} M), (c) $[\{Cu(im)Cu\}L']^{3+}$ (10^{-3} M) + N_3^- (0.1 M).

Table 1 EPR data for the two inequivalent copper centers of the azide adduct of $[\{Cu(im)Cu\}L']^{3+}$

Parameter	Copper 1	Copper 2
$g_{\perp}$	2.07	2.06
$g_{\|}$	2.23	2.16
$A_{\|}/cm^{-1}$	490	490
$g_{\|}/A_{\|}/cm$	138	134

pure sample of the azide adduct of $[\{Cu(im)Cu\}L']^{3+}$ were unsuccessful, since the N_3^- derivative was prepared with a large excess of N_3^-. When a 1 : 1 solution of N_3^- and $[\{Cu(im)Cu\}L']^{3+}$ was prepared, a small amount of the N_3^- adduct was formed (trace b), as indicated by the slight difference between the spectra a and b at 320 mT.

Equilibrium data for azide binding

The addition of azide to solutions of the model compounds causes the growth of a moderately intense absorption band in the 350–410 nm range which characterizes the azide-to-metal charge transfer transition. The equilibrium constants K were determined according to the procedure described in the experimental section. Hill plots yield straight lines with slopes of $n = 1$ for the three complexes, establishing the formation of azide adducts with 1 : 1 stoichiometry. Representative spectral and titration data are given in Fig. 3. The optical features of the $N_3^- \rightarrow Cu(II)$ LMCT bands and the equilibrium K values are summarized in Table 2. For the complexes $[\{Cu(im)Cu\}L']^{3+}$ and $[Cu_2L]^{4+}$, the equilibria of the azide

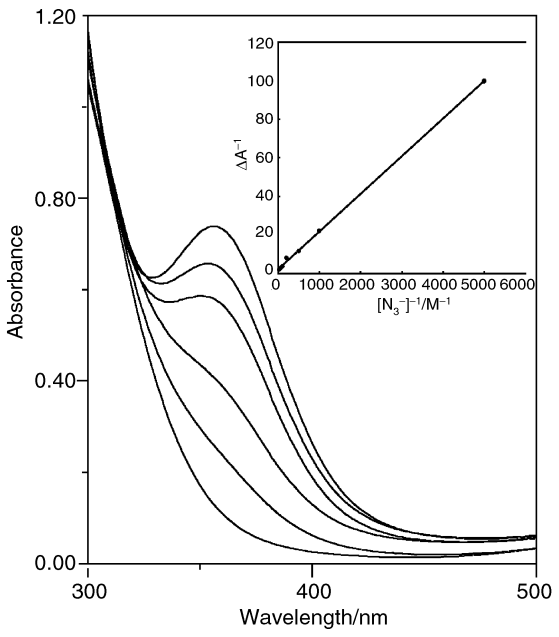


Fig. 3 UV-visible spectra recorded as a function of $[N_3^-]$ for addition of azide to $[\{Cu(im)Cu\}L']^{3+}$ (2×10^{-4} M, pH = 7.5, $I = 0.2$ M), from bottom to top: $[N_3^-] = 0.0$, 0.005, 0.01, 0.03, 0.07 and 0.10 M. The insert shows a plot of $1/\Delta A$ as a function of $1/[N_3^-]$.

Table 2 UV-visible spectral parameters and equilibrium constants for the azide adducts of the dicopper complexes

	$[\{Cu(im)Cu\}L']^{3+}$	$[\{Cu(im)Cu\}L]^{3+}$	$[Cu_2L]^{4+}$
λ_{max}/nm	364	405	400
$\varepsilon/M^{-1}\ cm^{-1}$	2500	3200	3050
K	105 ± 5	270 ± 15	14000 ± 500

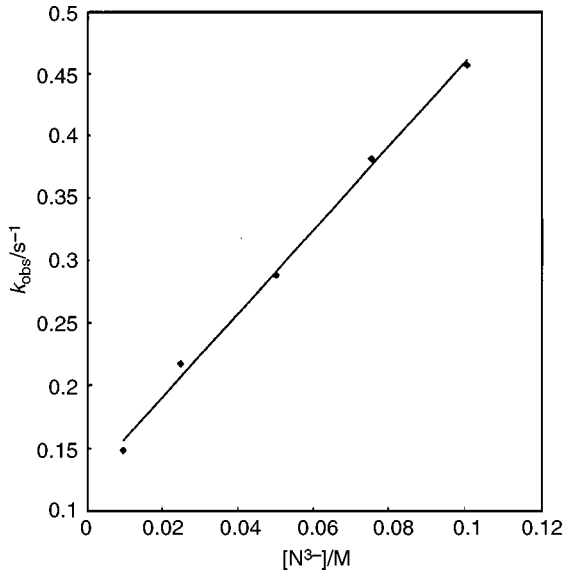


Fig. 4 k_{obs} (s^{-1}) as a function of [N_3^-] (M) for the addition of N_3^- to $[Cu_2L]^{4+}$ (2.5×10^{-4} M, pH = 7.5, I = 0.2 M).

binding was quickly reached, whereas for $[\{Cu(im)Cu\}L]^{3+}$ incubation of the mixtures at 40 °C for several days was necessary to reach equilibrium before the spectroscopic measurements.

Kinetic studies

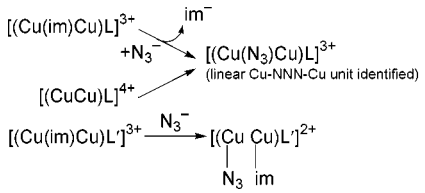
The kinetics of N_3^- adduct formation was investigated by a stopped-flow spectrophotometric method under pseudo-first-order conditions [N_3^-] ≫ [complex] at the wavelength corresponding to the ligand-to-metal charge transfer transition (λ between 364 and 405 nm). The absorbance change with time showed a single exponential curve for the three complexes, indicating that the N_3^- adduct formation reaction proceeds through a single rate-limiting step. For the complex $[\{Cu(im)Cu\}L]^{3+}$ binding of the anion was slow, so that the kinetics of the azide addition was investigated by classical spectrophotometric measurements. The corresponding pseudo-first-order rate constants k_{obs} (in s^{-1}) exhibited a linear variation as a function of the concentration of N_3^- with nonzero intercepts (Fig. 4 shows $[Cu_2L]^{3+}$ as an example). This is interpreted by a rate-determining step with rate constants k_f and k_r for the forward and reverse reactions related by the equation:

$$k_{obs} = k_f[N_3^-] + k_r$$

The values of k_f and k_r are reported in Table 3.

Discussion

The equilibrium constants for azide binding indicate a significantly higher affinity of the azide ion for $[Cu_2L]^{4+}$ than for the two imidazolate-bridged dicopper complexes. This can be partly attributed to the higher charge of this complex (4+ *vs.* 3+). For all the complexes, the results are in agreement with the addition of one azide ion. For $[\{Cu(im)Cu\}L']^{3+}$, the inequivalence of the two uncoupled copper centers, as shown by the EPR spectrum of the azide adduct, suggests monodentate coordination with one azide and one imidazolate. It can be proposed that the binding of the azide causes cleavage of one of the copper–imidazolate bonds. Characterization of the imidazolate-to-copper(II) LMCT band is difficult, since it is overlapped by a band due to the ligand. For the complexes $[Cu_2L]^{4+}$ and $[\{Cu(im)Cu\}L]^{3+}$, the azide adduct exhibits the same spectroscopic features as those for the linear μ-azido-bridged dicopper cryptate,[16] suggesting the release of the imidazolate ligand from $[\{Cu(im)Cu\}L]^{3+}$. It is emphasized that the macrobicyclic structure favors the formation of collinear Cu–NNN–Cu or $Cu–N_{im}N_{im}–Cu$ geometries, as confirmed by X-ray, UV-visible spectrophotometry and magnetic data.[16] The proposed reaction pathways are summarized in Scheme 2.



Scheme 2 Summary of the reactions of azide with SOD model compounds.

The values of the ratio k_f/k_r {4.4, 2.1 and 28.2 for $[\{Cu(im)Cu\}L']^{3+}$, $[\{Cu(im)Cu\}L]^{3+}$ and $[Cu_2L]^{4+}$, respectively} are very different from the equilibrium constant K. Indeed, if the azide binding process occurs in one step, one would obtain $K = k_f/k_r$. Thus, our kinetic results show that the azide binding can be separated into several elementary steps. An initial fixation of the azide ligand can be proposed in the rate-limiting step for the three complexes, followed by the non-rate-determining cleavage of the imidazolate bridge in the case of $[\{Cu(im)Cu\}L']^{3+}$ and $[\{Cu(im)Cu\}L]^{3+}$. A comparison of the kinetic data allows the influence of the structure of the ligand (macrocycle or macrobicycle) and of the coordination sphere to be discussed. The fixation of N_3^- has been found to be rapid for the complexes $[\{Cu(im)Cu\}L']^{3+}$ (k_f = 6.3 s^{-1} M^{-1}) and $[Cu_2L]^{4+}$ (k_f = 3.4 s^{-1} M^{-1}) with the copper ion tetra-coordinated to the ligand. In this case, assuming that one or two coordinating sites are occupied by a water molecule, the determining step could thus involve the substitution of a water molecule by an N_3^- anion. The rate constant values are consistent with labile water molecules as generally observed for Cu(II) complexes.[19] Further studies would be required to demonstrate whether the mechanism is associative or dissociative. A dissociative mechanism would involve the detachment of a water molecule in the rate-determining step, which is very fast in copper complexes.[19] The values of k_f suggest instead that the transition state of the rate-determining step is the formation of the $Cu–N_3^-$ bond. The rate constant obtained for $[Cu_2L]^{4+}$ suggests that the penetration of the incoming ligand is not hindered by the tight structure of the macrobicycle. So the very slow kinetics observed for the macrobicyclic complex $[\{Cu(im)Cu\}L]^{3+}$ ($k_f = 1.25 \times 10^{-5}$ s^{-1} M^{-1}) where Cu(II) is penta-coordinate (five nitrogen atoms from L and imidazolate) can be ascribed to the absence of a labile coordination site.

In summary, our studies have established interesting structural effects for the reactivity of N_3^- with our complexes. The EPR study provides information on the reaction product: the formation of an N_3^- bridge between the two metal centers is favored in the macrobicyclic ligand while N_3^- is coordinated

Table 3 Kinetic data for the addition of azide to the dicopper complexes

	$[\{Cu(im)Cu\}L']^{3+}$	$[\{Cu(im)Cu\}L]^{3+}$	$[Cu_2L]^{4+}$
k_f/s^{-1} M^{-1}	6.3 ± 0.3	(1.25 ± 0.06) × 10^{-5}	3.4 ± 0.1
k_r/s^{-1}	1.44 ± 0.02	(0.59 ± 0.02) × 10^{-5}	0.12 ± 0.01

to one metal in the complex with the macrocyclic ligand. The kinetic study provides information on the rate-determining step, describing the incipient coordinating bond (copper–azide nitrogen). It has been shown that fast fixation of N_3^- requires a labile leaving group, as observed for the complexes containing a Cu(II) tetra-coordinated to the ligand in contrast to the complex with a penta-coordinate Cu(II). The crystal structure of the azide-inhibited bovine Cu,Zn SOD evidences the direct coordination of the azide anion to Cu(II), in place of the metal-bound water molecule, without breaking of the imidazolate bridge.[11] The noticeable difference between our models and the enzyme arises from the fact that the imidazolate is bound to the protein chain and is maintained in close proximity to the metal centers after azide fixation. Nevertheless, the kinetic data obtained with the biomimetic models evidence that labile coordination is required to enhance the rate of fixation of N_3^-, as for the enzyme. This finding is most relevant for biomimetic models of SOD.

Experimental

Materials

The Cu(im)Cu and CuCu complexes have been previously described by us.[14,15] An authentic sample of $[\{Cu(N_3)Cu\}L](ClO_4)_3$ was been prepared according to ref. 16. Commercial reagents were used as obtained without further purification. Solvents were purified by standard methods before use.

Caution. Although no problems were encountered during the preparation of the perchlorate salts described below, suitable care and precautions should be taken when handling such potentially hazardous compounds.

Spectroscopic measurements

EPR spectra were recorded at 100 K on a Bruker ER 100 D spectrometer operating at 9.4 GHz with 2,2-diphenyl-1-picrylhydrazyl as an external calibrant. The pH of the aqueous solution was adjusted before the addition of dimethylsulfoxide (DMSO) to obtain a 1 : 1 v/v water–DMSO solution, which was then cooled to 100 K. UV-visible spectra were obtained on a Perkin Elmer Lambda 2 spectrophotometer with quartz cells at room temperature.

Azide titrations and data analysis

Azide binding studies were performed by adding concentrated aqueous solutions of sodium azide to 2×10^{-4} M solutions of the complexes in the same solvent. The ionic strengh of the solutions was adjusted to 0.2 M with sodium chloride and the pH was fixed at 7.5 with a 0.05 M Hepes buffer. The sodium azide concentrations varied from 2×10^{-4} to 0.1 M. The equilibrium constant for the copper–azide adduct can be obtained according to the following equation, assuming 1 : 1 stoichiometry:

$$K = [CN_3]/[C][N_3] = \Delta A/(\Delta A_\infty - \Delta A)[N_3]$$

where [C], $[CN_3]$ and $[N_3]$ represent the concentrations of copper complex, azide adduct and free azide, respectively. ΔA is the absorbance change at the λ_{max} of the LMCT band induced by the addition of azide and ΔA_∞ is the absorbance change for complete formation of the azide adduct (at infinite ligand dilution). This equation can be modified as follows:

$$1/\Delta A = (1/K\Delta A_\infty)(1/[N_3]) + 1/\Delta A_\infty$$

A plot of $1/\Delta A$ against $1/[N_3]$ should thus yield a straight line with a slope of $1/K\Delta A_\infty$ and x and y intercepts of $-K$ and ΔA_∞, respectively. Formation of 1 : 1 stoichiometry adducts was established by the use of the Hill equation:

$$\log[\Delta A/(\Delta A_\infty - \Delta A)] = n \log[N_3] + \log K$$

The value of ΔA_∞ used in this equation is the absorbance change obtained at high azide complex ratios, when saturation of the ligand binding sites was observed. A plot of $\log[\Delta A/(\Delta A_\infty - \Delta A)]$ against $\log[N'_3]$ should yield a straight line with slope $n = 1$ in the case of simple binding of the azide anion to the copper complex. For the complex $[\{Cu(im)Cu\}L]^{3+}$ the equations were modified, since the reaction leads to two products: $[\{Cu(N_3)Cu\}L]^{3+}$ and im^-.

Kinetic studies

Kinetic measurements were performed with a Kinspec UV (Bio-Logic Co., Claix, France) stopped-flow spectrophotometer equipped with a diode array detector (J & M) and connected to a Tandon microcomputer. The kinetic data were treated on-line with the commercial BIO-KINE program (Bio-Logic Co.). The ionic strength was fixed at 0.2 M (NaCl) and the pH was fixed at 7.5 with a 0.05 M Hepes buffer. Formation kinetics were carried out under pseudo-first-order conditions at 25 °C with N_3^- in excess with respect to the complex. The concentration of azide was varied from 10^{-2} to 10^{-1} M whereas the concentration of complex was fixed at 2.5×10^{-4} M. In every case, first-order kinetics was observed. The reported rate constants are the average of 8 replicate determinations.

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