indicating that the Mg^{2+} and Ca^{2+} complexes of pyrophosphate are different (Calvo, 1965, 1968; Mildvan, 1970). This difference is likely to extend to their respective ATP complexes and to their interactions with myosin. Similar differences in reactivity of the thiols in the Ca^{2+} M** and Mg^{2+} M** states can also be detected with the bifunctional reagent *p*-phenylenedimaleimide as a conformational probe (Burke and Reisler, unpublished results). Differences in the Ca^{2+} and Mg^{2+} steady-state complexes of myosin ATPase are also detected by oxygen exchange. The presence of Ca^{2+} completely prevents oxygen exchange whereas at comparable turnover times in the presence of Mg^{2+} (actomyosin) significant incorporation is observed (Shukla and Levy, 1977).

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Proton Relaxation Study of the Hog Kidney Diamine Oxidase Active Center†

Michael D. Kluetz*,‡ and Paul G. Schmidt

ABSTRACT: Proton relaxation studies of the interactions with hog kidney diamine oxidase of water, substrate-analogue inhibitors, and product analogues indicate that the active site Cu(II) is *not* located near the oxidizing site of the enzyme, rather near the nonoxidized end of the binding substrate. The studies with histamine derivatives provide evidence for a concentration-dependent occupation of two sites. The site which is populated at high concentrations provides proximity of the imadazole ring nitrogen N_1 to the Cu(II). Water binds at the Cu(II) of the native enzyme. However, this water is probably not involved in the hydrolysis of the enzyme-substrate imine bond to eliminate the first reaction product. O_2 does not compete with H_2O for a site on the Cu(II) ion. In the case of one of the probes, namely the ammonia (product) analogue dimethylamine, the validity of the protein relaxation results was verified by also observing the nitrogen (^{15}N) relaxation rates of ammonia itself. The conclusion that the ammonium ion is not directly bonded to the active site Cu(II) is reached from both the proton and nitrogen relaxation experiments.

The role of diamine oxidase (diamine:O_2 oxidoreductase (deaminating); EC 1.4.3.6) in the catabolism of various amines found in association with DNA in addition to its function in one of the catabolic pathways of histamine has led to considerable interest in this system. The excellent recent series of kinetic studies by Bardsley and co-workers (Bardsley and Ashford, 1972; Bardsley and Hill, 1970; Bardsley et al., 1970, 1971, 1972, 1973) together with the identification of the two prosthetic groups of the enzyme as Cu(II) (Mondovi et al., 1967a) and pyridoxal phosphate (Mondovi et al., 1967b) has produced a significantly greater understanding of the mode of action of the enzyme. However, many of the details of the catalytic mechanism are still unknown, Of these, the role of the metal ion is of particular interest.

The overall reaction catalyzed by diamine oxidase is

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$$RCH_2NH_2 + H_2O + O_2 \longrightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

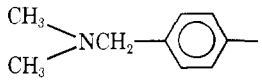
where R =

$NH_2CH_2CH_2CH_2-$

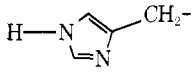
putrescine

$NH_2CH_2CH_2CH_2CH_2-$

cadaverine



p-dimethylaminomethylbenzylamine; DAB[1]



histamine

The aldehyde oxygen originates with the water, not the molecular oxygen.

The existence of a visible absorption band in the 410-nm region makes it likely that pyridoxal phosphate is bound through a Schiff's base linkage to a protein residue possessing a terminal amino group, such as lysine. Based on this and the extensive knowledge concerning pyridoxal chemistry and catalysis it is reasonable to suspect that the reaction proceeds through a mechanism of the type shown in Figure 1. Upon binding of the amine substrate a transimination occurs generating a Schiff's base linkage to the substrate which is then hydrolyzed to form the first product, the aminoaldehyde; subsequent binding of O_2 regenerates the pyridoxal phosphate–protein imine bond from the pyridoxamine form of the enzyme with release of NH_3 and conversion of the O_2 to H_2O_2.

We have undertaken to determine the role of the Cu(II) ion in this catalytic scheme. While there is evidence that the chelation of the metal in the enzyme by addition of EDTA, reduction of the Cu(II) to Cu(I), and removal of the metal with diethyldithiocarbamate all destroy the activity of the enzyme, it has as yet not been demonstrated that the Cu(II) actually performs a catalytic function. To attack this problem we have partially mapped the active center of the enzyme with respect to the Cu(II) by determining which of the substrates and products (or which parts of them) bind directly to or near the Cu(II) and have interpreted these results in light of the chemistry which is already known. The probe molecules used are various methylated histamines, which are substrate analogues, water (one of the reactants), and dimethylamine, an analogue of the product ammonia. Distances of various protons on these probe molecules from the Cu(II) were estimated making use of the large nuclear relaxation effect due to unpaired electron spin on the ion. We also utilized ^{15}N NMR to study directly the binding of ammonia.

Materials and Methods

Diamine Oxidase. The enzyme was purified by a procedure developed in our laboratory. The activity of the enzyme was measured by spectrophotometrically monitoring the oxidation of DAB; the procedure is an improvement of that described by Bardsley et al. (1972). The enzyme was electrophoretically pure with a specific activity near unity. Solutions of between 15 and 25 mg/mL enzyme were used in the NMR experiments. Any loosely bound excess metal was removed by dialysis against 0.05 M P_i, pH 7.2, containing approximately 0.5 mM EDTA followed by exhaustive dialysis against the same buffer without EDTA. The solutions were then lyophilized to dryness, redissolved in D_2O, and repeatedly dialyzed against 0.05 M P_i in D_2O. Protein concentrations were estimated by a biuret procedure as described by Goa (1953). Two Cu(II) ions are firmly bound to the holoenzyme of molecular weight 175 000 (Kluetz, 1975). Concentrations of the enzyme are reported in terms of one active center per 91 000 daltons.

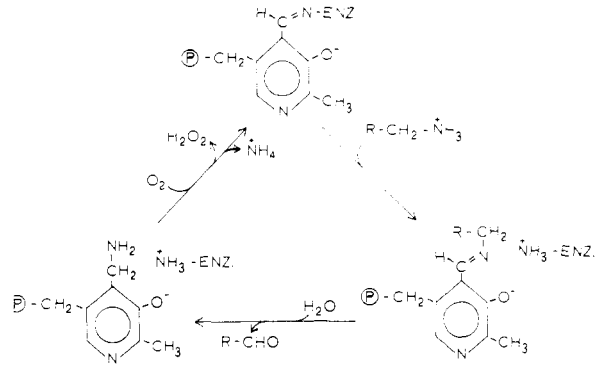
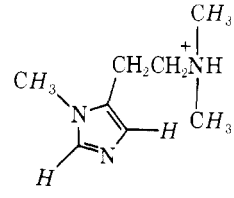
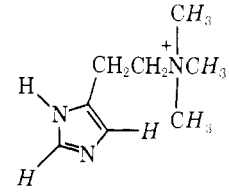


FIGURE 1: Diamine oxidase reaction. Depicted is the probable sequence of events in the oxidative deamination of an amine by diamine oxidase, employing the enzyme-bound pyridoxal 5′-phosphate.

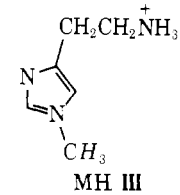
Substrates and Analogues for NMR. For these studies, resonances on the following molecules were monitored: *H*DO, $(CH_3)_2ND_2^+$, $^{15}NH_4^+$, 3-methyl-4(5)-(β-dimethylammonium)ethylimidazole chloride (MH I), 4(5)-(β-trimethylammonium)ethylimidazole chloride (MH II), and 1-methyl-4(5)-(β-ammonium)ethylimidazole chloride (MH III). The cationic structures of MH I, MH II, and MH III show the resonances in question. MH I and MH II were prepared by



MH I



MH II



MH III

applying the general methylation procedure of Cope et al. (1960) to histamine, utilizing methyl iodide. The products were formed in low yield (~10%) and were isolated by repeated crystallization from ethanol, methanol, and finally water. MH I apparently loses water of crystallization near 100 °C and decomposes around 206 °C. MH II melts between 228 and 230 °C in agreement with results previously reported. Both compounds appear to be somewhat light sensitive.

MH III was purchased from Calbiochem., Inc. Assignment of structures to MH I and MH II was facilitated by the NMR spectra of the methyl groups. MH I exhibited a single methyl resonance corresponding to two methyl groups at 3.4 ppm and a singlet corresponding to one methyl group at 3.5 ppm. MH III showed a singlet corresponding to three methyl groups at the same position as the resonance of the two groups in MH I, confirming their assignment as the amino methyls. The second methyl resonance of MH I is assigned to a methyl on

[1] The abbreviations used are: DAB, *p*-dimethylaminomethylbenzylamine; NMR, nuclear magnetic resonance; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid.

the imidazole N_3, as the methyl on the N_1 of MH III occurred much farther downfield (4.1 ppm). Dimethylamine was from Eastman. The water resonance was from residual protons in the buffer made from 99.8 atom % (D) D_2O (Stohler Isotopic Chemicals). All amine inhibitor solutions were approximately 0.5 M stock solutions in 0.05 M P_i–D_2O buffer; the D_2O for the buffer was made free of metal ions by passage through a 1 × 10 cm column of Chelex 100 (Bio-Rad Laboratories).

Proton Relaxation Measurements. Longitudinal Relaxation Times. T_1 was measured at 100 MHz (Varian HA-100) and 220 MHz (Varian HR-220) by the $T_{1\rho}$ method of Sykes and Wright (1970) for 100 MHz and London and Schmidt (1974) for 220 MHz.

Transverse Relaxation Times. T_2^{-1} was estimated from line widths at 60 MHz (Varian A60/A), both line width and $T_{1\rho}$ measurements at 100 MHz, and from $T_{1\rho}$ at 220 MHz. The $T_{1\rho}$ measurements involved a rapid adiabatic exponential sweep into the center of resonance (time constant <0.2 s) from about 100–200 Hz off-resonance with the phase sensitive detector tuned 90° from the usual absorption mode. Power levels of H_1 were generally 1 mG at 100 MHz and 2 to 3 mG at 220 MHz. These levels are sufficiently low that $T_{1\rho} = T_2$. The T_1 measurements involved (a) saturating the signal by remaining on resonance, (b) pulsing off resonance for a time, τ, then (c) adiabatically sweeping back on resonance to sample the extent of return of magnetization along the Z axis toward its equilibrium value. Estimated experimental uncertainty in the $T_{1\rho}$ measurements is ±5% and ±10% in the T_1 values.

Nitrogen-15 Relaxation Measurements. $(^{15}NH_4)_2SO_4$ (99 atom %) was used to help alleviate signal-to-noise problems, along with the pulse Fourier transform technique. Spectra were obtained on a Varian XL-100 spectrometer, operating at 10.1 MHz. A capillary of D_2O provided the lock signal. Approximately 50 000 accumulations of the signal were obtained for each measurement. The measurements were done at the Los Alamos Scientific Laboratory, through the courtesy of Drs. R. E. London and N. A. Matwiyoff; the labeled ammonium sulfate was a gift to them of Dr. Max Goldblatt.

Heteronuclear Decoupling of Nitrogen-14. In the case of nitrogen-bound methyl groups we found that the resonances were broader than expected (1.3 Hz for the ring methyls and 1.5 Hz for the amino methyls). Line widths for these peaks of MH I were reduced to ca. 0.9 Hz by irradiation at the ^{14}N resonance frequency. The standard HA-100 probe was modified for multinuclear heteronuclear decoupling after the method of Burton and Hall (1970). One-half watt was sufficient power for full decoupling. T_2's for the nitrogen-bound methyl groups in the presence of enzyme were routinely determined by line-width measurements with ^{14}N decoupling.

Nuclear Relaxation Induced by Paramagnetic Ions. Relaxation of nuclei near a paramagnetic ion is often dominated by the dipolar interaction between the nuclear spin and the unpaired electron spin, with some contribution from the scalar or hyperfine interaction. The dipolar contribution is highly distance dependent and allows evaluation of metal ion–nuclei distances of approach (Mildvan and Cohn, 1970).

The nuclear relaxation rates are given by the Solomon–Bloembergen equations:

$$1/T_{1m} = (2/15)\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + (2/3)S(S+1)(A/\hbar)^2\left(\frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (2)$$

$$1/T_{2m} = (1/15)\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6} \times \left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) + (1/3)S(S+1)(A/\hbar)^2\left(\tau_e + \frac{\tau_e}{1+\omega_s^2\tau_e^2}\right) \quad (3)$$

where γ_I is the magnetogyric ratio of the nuclear spin I, g is the Lande g factor of the electron, S is the total electron spin, β is the Bohr magneton, r is the distance of separation of the nucleus and the paramagnetic center, A is the scalar (hyperfine) coupling constant between the nucleus and the unpaired electron spin, and ω_I and ω_s are, respectively, the resonance frequencies of the nucleus and electron.

τ_c and τ_e are the correlation times describing the motions which modulate the dipolar and the scalar interactions, respectively, and are the composite quantities defined below:

$$1/\tau_c = 1/\tau_s + 1/\tau_m + 1/\tau_r \quad (4)$$

$$1/\tau_e = 1/\tau_s + 1/\tau_m \quad (5)$$

where τ_s is the electronic relaxation time, τ_m is the exchange lifetime and τ_r is the rotational reorientation time. The overall correlation time will be determined by the most rapid process. We expect τ_s to be on the order of a few nanoseconds possibly increasing at high NMR frequency (vide infra); τ_r is probably the overall reorientation time of the macromolecule, which for a protein of molecular weight 175 000 will be on the order of 100–150 ns. Exchange lifetimes are generally longer than the electronic relaxation time especially for the substrate analogues other than water used in this study.

The spectral density terms in ω_s in the Solomon–Bloembergen equations will be negligible for proton frequencies on the order 60–220 MHz, whereas $\omega_I^2\tau_c^2$ can be near unity. One can also neglect the scalar term in the $1/T_{1m}$ equation. In the case of $1/T_{2m}$ the second spectral density term in the scalar contribution is negligible, but there remains a contribution of $(A'/2)(\tau_e)$. For Cu(II)–ligand systems this latter term cannot be ignored and will be discussed further.

Information on $1/T_{1m}$ and $1/T_{2m}$ is obtained by measuring the relaxation rates of nuclei on small molecules exchanging from sites on the macromolecule. For diamine oxidase ligands a simple binding scheme suffices as a first approximation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \quad (6)$$

where E and I are the macromolecule and small molecule respectively, and k_1 and k_{-1} are the rate constants for the formation and dissociation of the complex, EI.

If this small molecule is present in large excess over the concentration of binding sites (the present case) the relaxation rates of nuclei on the small molecules are given by (Swift and Connick, 1962; Luz and Meiboom, 1964):

$$1/(T_2)_{ex} = 1/(T_2)_{obsd} - 1/(T_2)_f = (P/\tau_m)\frac{\left[\frac{\tau_m}{T_{2m}}\left(1+\frac{\tau_m}{T_{2m}}\right) + \tau_m^2\Delta^2\right]}{[(1+\tau_m/T_{2m})^2 + \tau_m^2\Delta^2]} \quad (7)$$

$$1/(T_1)_{ex} = 1/(T_1)_{obsd} - 1/(T_1)_f = \frac{P}{T_{1m}+\tau_m} \quad (8)$$

where $P = [EI]/[I]$ is the fraction of small molecule bound, $\tau_m = 1/k_{-1}$ is the average lifetime of the complex, Δ is the chemical-shift difference (in radians per second) between free and bound environments, and $1/(T_1)_f$ and $1/(T_2)_f$ are the

TABLE I: Bound Relaxation Rates and Dissociation Constants of Various Diamine Oxidase Probe Molecules.[a]

	T_{2m}^{-1} (s^{-1})	T_{1m}^{-1} (s^{-1})	K_D (M)
HDO (two per subunit)	260 000	54 000 35 000 (220)	
Dimethylamine	360 190 (220)	75 45 (220)	$82 \pm 10 \times 10^{-3}$
MH I			
Ring methyl	200		
Amino methyl	70		
MH III			
Methyl	40	17	$<30 \times 10^{-3}$
MH I, II (site 1)			
H_2	400		
H_4	360		
MH I (site 2)			
H_2	45 000		
H_4	60 000		

[a] Frequency = 100 MHz unless indicated otherwise in parentheses.

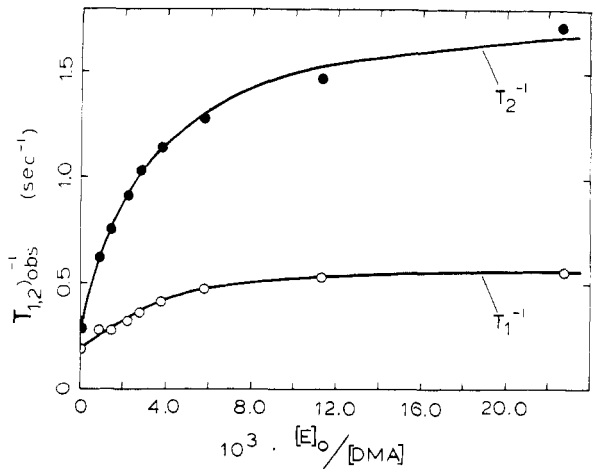


FIGURE 2: Dimethylamine relaxation rates. Relaxation rates T_1^{-1} and T_2^{-1} at 100 MHz, 30 °C (both measured by the $T_{1\rho}$ technique), of the methyl protons of dimethylamine are plotted vs. the enzyme/amine ratio, in the presence of 0.13 mM diamine oxidase. The data at "infinite" concentration (zero fraction bound) were obtained in the absence of enzyme. The relaxation rates in this figure are the observed rates, uncorrected for the free dimethylamine rates (the ordinate intercepts).

nuclear relaxation rates for the free species. If $\tau_m^2\Delta^2 \ll \tau_m/T_{2m}\,(1 + \tau_m/T_{2m})$, then

$$1/(T_2)_{ex} = \frac{P}{T_{2m} + \tau_m} \tag{9}$$

Under these conditions, one can extract the bound relaxation rates and hence determine distances if chemical exchange is faster than nuclear relaxation in the bound state, when the equations reduce further to

$$1/(T_i)_{ex} = \frac{P}{T_{im}} = (1/T_{im})\frac{[E]_0/[I]}{1 + K_D/[I]} \tag{10}$$

where $i = 1, 2$, K_D is the dissociation constant of the EI complex, and we have employed the scheme of eq 6 to determine P in terms of $[E]_0/[I]$.

The validity of this limit is established by measuring the temperature dependence of the relaxation, which will reflect the rate-limiting process and verify that $1/(T_i)_{ex}$ is governed by relaxation in the bound state, as indicated in eq 10. If either a τ_m or a $\tau_m^2\Delta^2$ term were very large, one would see a decrease in $1/(T_1)_{ex}$ or $1/(T_2)_{ex}$ with decreasing temperature due to an increase in τ_m. In addition, this dependence would be governed by a relatively large apparent activation energy, on the order of 10 kcal/mol. However, if the limit of eq 10 is obtained, we would observe the temperature dependence of the bound relaxation, which in turn is governed by the temperature dependence of the correlation time, τ_c. As mentioned above, we expect this correlation time to be dominated by the electronic relaxation time, τ_s. While this temperature dependence can be in either direction, it is easily distinguished from that of exchange processes by its substantially smaller activation energy, on the order of 1 kcal/mol or less.

Results

Interaction of Diamine Oxidase with Dimethylamine. It is known from kinetic studies that NH_3 is a diamine oxidase product-inhibitor with a K_i of 34 mM (Bardsley et al., 1973). However, it is not possible to observe the protons of NH_4^+ in D_2O or in H_2O because of their rapid exchange with the solvent. A reasonable analogue of NH_3 is dimethylamine, a compound particularly well suited for NMR relaxation studies due to the presence of six equivalent protons, thereby reducing the minimum accessible concentration.

Aliquots of a concentrated dimethylamine solution were added stepwise to a 24 mg/mL solution of diamine oxidase; the concentration of the product analogue ranged from 11 mM to 177 mM. The results at 100 MHz are shown in Figure 2, where the observed $1/T_1$ and $1/T_2$ are plotted vs. the reciprocal amine concentration.

The data were fit through the use of a computer program to the binding scheme of eq 6. Best fits occurred using dissociation constants of 90 mM for T_2 and 74 mM for T_1; this discrepancy in K_D is probably within the uncertainty of the fit. The computer fit also yields the bound relaxation rates $1/T_{1m}$ and $1/T_{2m}$ which are collected in Table I. Relaxation measurements were made at 220 MHz for a single dimethylamine concentration, 22 mM, and these data are included in Table I.

Interaction of Diamine Oxidase with $^{15}NH_4^+$. One of the problems of using dimethylamine as an analogue of ammonia is that the distance of the nitrogen atom from the paramagnetic center is not obtained, but rather an average distance of two methyl groups. This leads to ambiguity in the nitrogen position. The availability of isotopically enriched $^{15}NH_4^+$ made feasible

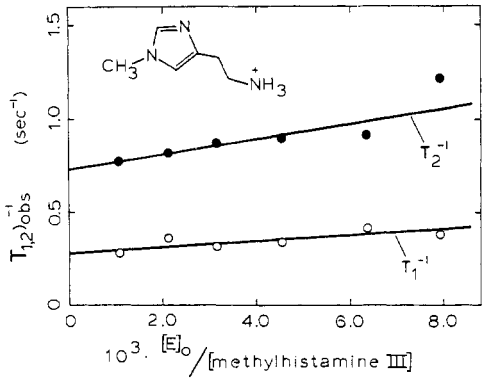


FIGURE 3: MH III relaxation rates with diamine oxidase. Relaxation rates T_1^{-1} and T_2^{-1} at 100 MHz, 30 °C (both measured by the $T_{1\rho}$ technique), of the methyl protons of MH III are plotted vs. the ratio of the initial enzyme and amine concentrations. The initial enzyme concentration was 0.12 mM. The relaxation rates are uncorrected for free MH III relaxation rates.

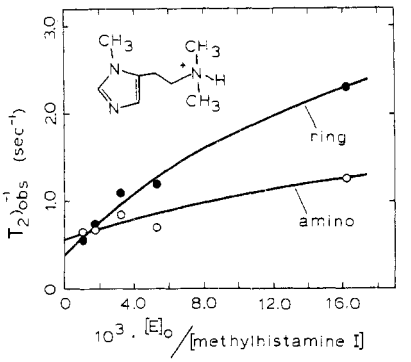


FIGURE 4: MH I relaxation rates. Shown are the observed relaxation rates, $T_2^{-1}{}_{obsd}$, at 100 MHz, 30 °C (measured from linewidths with ^{14}N decoupling), of the methyl protons of MH I vs. the enzyme/amine ratio, in the presence of 0.12 mM diamine oxidase. Both the ring and amino methyl groups were observed.

the direct observation of nitrogen in dilute ammonia–diamine oxidase solutions.

^{15}N has a nuclear spin of $^1/_2$ and in ammonia is scalar coupled to four protons. But the splitting is not observed due to rapid exchange of the protons with H_2O at pHs near 7 to yield a sharp singlet nitrogen signal.

Line widths of 87 mM $^{15}NH_4^+$ with and without 0.1 mM diamine oxidase and of 166 mM $^{15}NH_4^+$ with 0.1 mM enzyme were indistinguishable within experimental uncertainty. The average line width was approximately 2 Hz.

Since it is known that the ammonia dissociation constant is 34 mM, we can calculate that a substantial fraction of the enzyme sites were occupied in the NMR measurements. The lack of any measurable contribution of the enzyme to nitrogen relaxation indicates that ammonia does not bind near the Cu(II). It is important to point out that, compared with protons, the effective range of distance which will yield measurable effects on the nitrogen line width is substantially reduced because of the smaller magnetogyric ratio for ^{15}N (eq 3). A nitrogen-15 nucleus must be 2.2 times closer to the paramagnetic center to see the same effect one would see with a proton. However, it appears certain that the nitrogen is not within bonding distance of the Cu(II); calculating the effect at this distance and with the fraction bound, the observed line width would be 10 Hz, an easily measurable quantity.

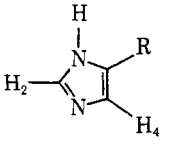
Binding of 1-Methyl-4(5)-(β-ammonium)ethylimidazole Chloride (MH III). MH III was added stepwise to a 24 mg/mL solution of diamine oxidase and both T_1 and T_2 of the methyl protons were measured at 100 MHz. The observed effect was small; the data are best fit by straight lines of small slope. It is known from kinetic studies that histamine methylated at both ring nitrogens binds as well to diamine oxidase as does histamine (Bardsley et al., 1971). It is likely then that MH III also binds sufficiently well that, even at the lowest concentration used in this experiment, the enzyme was saturated. The relaxation effect would thus be linear in $[MH\ III]^{-1}$, as the data seem to indicate. Therefore, while no dissociation constant was determinable (except to say that it must be less than 30 to 40 mM), bound relaxation rates were obtained from the slope of the curves in Figure 3. Least-squares analyses of these curves yield values for $1/T_{1m}$ and $1/T_{2m}$, which again are presented in Table I.

3-Methyl-4(5)-(β-dimethylammonium)ethylimidazole Chloride (MH I) and 4(5)-(β-Trimethylammonium)ethylimidazole Chloride (MH II). a. MH I Methyl Protons. The ring and amino methyl peaks of MH I are well resolved and substantial broadening of either peak can be tolerated before overlap becomes a problem. As it is very difficult to measure $T_{1\rho}$ in the presence of neighboring peaks, it was necessary to use line-width measurements to determine T_2; T_1 was not determined in these experiments.

MH I is a particularly good probe of diamine oxidase. The location of methyl groups on both ends of the substrate analogue allows two sites to be monitored at once. The loss of reactivity upon methylating the amino group is also advantageous.

A preliminary experiment indicated that effects on the methyls were not particularly large; it was also noted that the line widths of the methyls in the absence of enzyme were excessive (on the order of 1.5 Hz). Methyl groups on small molecules generally have line widths of less than 1 Hz. The extra broadening for MH I is due to scalar coupling of the methyl protons with the quadrupolar ($I = 1$) N-14 nucleus. Decoupling at the N-14 resonance frequency markedly decreased the line widths (see Materials and Methods). T_2 measurements of these protons were made as MH I was titrated into a 20 mg/mL solution of diamine oxidase. To account for inhomogeneity broadening, line widths of protons of dilute *tert*-butyl alcohol were subtracted from those of the amine methyls. The line widths as a function of $[E]_0/[MH\ I]$ are shown in Figure 4. Estimates of bound relaxation rates from the initial slopes of these two curves indicate that the effect of the Cu(II) on both methyl groups was moderate (Table I). However, it was noted in the sample containing the highest concentration of MH I that the imidazole *ring* protons were quite broad. A complete study of this effect was therefore undertaken using both methylhistamines I and II.

b. MH I and MH II Ring Protons. The two carbon-bound ring protons of imidazole compounds have been used extensively as probes of macromolecular binding centers. Chemical shifts rather than broadening are most often studied. The two protons, referred to as H_2 and H_4, in D_2O give peaks in the



general imidazole structure

region 7–9 ppm from 2,2-dimethyl-2-silapentanesulfonate. H_2 is downfield of H_4 and, at the pH of our experiments (7.5), the two resonances are about 70 Hz apart. Each resonance represents an unresolved multiplet with line widths of about 3 Hz.

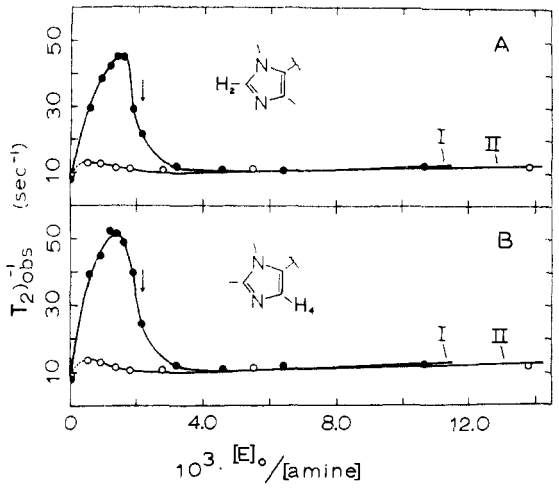


FIGURE 5: Diamine oxidase with MH I and MH II. The observed relaxation rates, $T_2^{-1}{}_{obsd}$, at 100 MHz, 30 °C (measured from linewidths), of the imidazole ring protons H_2 (A) and H_4 (B) of methylhistamines I and II are plotted vs. the enzyme/amine ratio, in the presence of 0.12 mM enzyme. (●—●) MH I; (○—○) MH II.

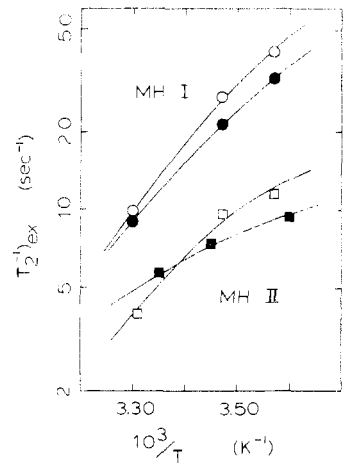


FIGURE 6: Temperature dependence of methylhistamine ring protons. The transverse relaxation rate of the ring protons of MH I and MH II in the presence of 0.12 mM DAO is plotted vs. the reciprocal temperature. The experiments were at 100 MHz; the amine concentrations used are indicated by the arrows in Figure 5. (●—●) MH I H_2; (○—○) MH I H_4; (■—■) MH II H_2; (□—□) MH II H_4.

Proton decoupling of one resonance reduced the line width of the other to about 1–1.5 Hz.

Observations of these proton line widths were made in the presence of diamine oxidase without decoupling. The peaks are markedly broadened in the presence of enzyme, and increases in the width of the multiplet provide a valid measure of changes in $1/T_2$. All spectra were obtained on the Varian HA-100 using a computer of averaged transients to enhance the signal-to-noise ratios; at the lowest concentrations of amine, 60 scans were accumulated. To check for extraneous broadening due to possible field drift during the accumulation process, several scans of the *tert*-butyl alcohol resonance were collected. The line width was still extremely small, indicating no problem with drift.

The results of addition of MH I to a solution of 20 mg/mL diamine oxidase and MH II to a 15 mg/mL enzyme solution are shown in Figure 5. The data for the H_2 protons and for the H_4 protons are shown in separate frames to compare the effects of the two compounds more closely. The results are strikingly different from the usual behavior as shown, for example, by dimethylamine (Figure 2). At low concentrations, where the maximum relaxation effect should be observed, virtually no effect is seen. However, at a concentration near 100 mM the relaxation rate rapidly becomes very large with increasing amine concentration and finally decreases toward the appropriate limit.

The immediate qualitative explanation of this result is that the amines are exchanging with at least two different sites. At low concentrations MH I and MH II bind to a relatively strong binding site which places the imidazole ring quite far from the Cu(II); at higher concentrations the methylhistamines bind additionally to a second, weaker, site which provides proximity of the ring to the metal ion.

The limiting slopes of the MH I curves at high concentrations of methylhistamine were used to estimate bound relaxation rates corresponding to the site in proximity to the Cu(II), while the data at low concentrations yields relaxation rates for the inhibitor in the stronger, initially occupied site (see Table I).

It is known that the addition of a third methyl group to the amino function of histamine greatly reduces its capacity to bind to diamine oxidase. This is presumably the reason for the reduced maximum relaxation effect seen with MH II; the relaxation effects are almost completely diluted out due to the small fraction bound before the concentration is high enough to cause MH II to bind to the second site near the Cu(II).

Temperature Dependences of Relaxation. Relaxation rates $1/(T_2)_{ex}$ and $1/(T_1)_{ex}$ were measured as a function of temperature for several of the amine inhibitors. The results, plotted in Figure 6, show that $1/(T_2)_{ex}$ and $1/(T_1)_{ex}$ decrease with increasing temperature consistent with the fast exchange limits of eq 7 and 8 such that $T_{1m}, T_{2m} > \tau_m$. For both $1/T_1$ and $1/T_2$ the observed activation energies are on the order of 1 to 2 kcal/mol. Some of the decrease in relaxation rates for dimethylamine and MH I at higher temperatures may be due to a decrease in the fraction bound since these compounds are not necessarily saturating their binding sites, but the effect would not be so large as to mask a dependence of relaxation rate on $1/\tau_m$.

Correlation Times for Dipolar Relaxation. Cu(II)-proton distances may be calculated from nuclear relaxation data if the electron-nuclear dipole-dipole effect is isolated and the correlation time, τ_c, is known. However, for several of the amine analogues $1/T_{2m}$ has potential contributions from (1) electron-nuclear scalar coupling and (2) nuclear-nuclear dipole-dipole interactions modulated by rotational reorientation. $1/T_{1m}$ is not subject to significant contributions from either of these processes.

Esperson et al. (1974) have made a detailed study of the scalar contribution to proton relaxation in Cu(II)-amine complexes (not macromolecular) including that between Cu(II) and imidazole. The scalar contribution was determined from the ratio $R = T_{1m}/T_{2m}$. Referring to the dipolar contribution as D ($= 1/T_1$), and the scalar part as S ($1/T_{2m} = D + S$), the percent contribution of scalar interactions to $1/T_{2m}$ is related to R by % $S = 100(R - 7/6)/R$. Esperson et al. (1974) found that, for Cu(II)-imidazole complexes, R was between 30 (for H_2) and 50 (for H_4 and H_5). However, if this complex is immobilized on a macromolecule, the scalar contribution, governed by τ_S just as in the small complex, remains the same, while the dipolar term increases by a factor of, perhaps, 200 due to the effective correlation time changing from τ_r ($\sim 1 \times 10^{-11}$ s) to τ_S ($\sim 2 \times 10^{-9}$ s; vide infra). Given this approximate correlation time change for the enzyme complex the contribution of S to $1/T_{2m}$ is less than 10%.

The diamagnetic component of $1/T_{2m}$ is more difficult to

TABLE II: Correlation Time τ_c (ns).

Method	Dimethylamine	MH III
T_{1m} vs. ω^2	0.8	
$T_{2m}^{-1}/T_{1m}^{-1})_{100}$	3.6	2.1
$T_{2m}^{-1}/T_{1m}^{-1})_{220}$	1.6	
Proton-Cu(II) Distances (Å)[a]		
MH I (site 2)		
H_2		4 ± 1
H_4		4 ± 1
MH I, II (site 1)		
H_2		8 ± 2
H_4		8 ± 2
MH I		
Ring methyl		9 ± 2
Dimethylamine		8 ± 2

[a]Calculated using an average correlation time of 2.5 ns.

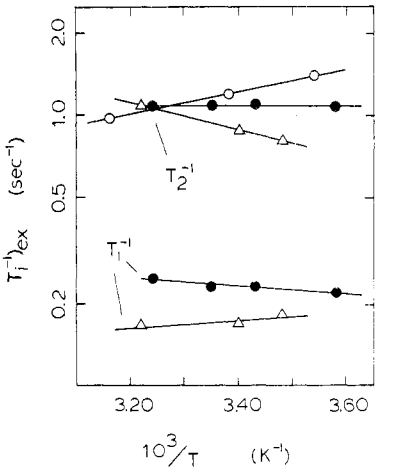


FIGURE 7: Temperature-frequency dependence of HDO relaxation. The exchange contribution to the relaxation rates of HDO (in the presence of 0.13 mM diamine oxidase) is plotted vs. the reciprocal temperature. T_1 and T_2 measurements were made at 100 and at 220 MHz by the $T_{1\rho}$ method; T_2 measurements at 60 MHz were from linewidths. (○—○) 60 MHz; (●—●) 100 MHz; (△—△) 220 MHz.

estimate, depending as it does on the degree of internal rotation which, for the methyl groups of the analogues used here, may be considerable. In any case the measured $1/T_{2m}$ is an upper limit on the paramagnetic contribution.

Estimates of the correlation time for dipolar relaxation may be made by two approaches: the NMR frequency dependence of $1/T_{1m}$ and the ratio of T_{2m}^{-1}/T_{1m}^{-1} (Mildvan and Cohn, 1970). Table II lists the results of these calculations for several analogues. Given the approximations involved in $1/T_{2m}$ the frequency dependence of $1/T_{1m}$ might be expected to yield a more reliable number. However, the correlation time might well be changing in the range 100–220 MHz. With dimethylamine and MH I values of τ_c range from 0.8 ns to 3.6 ns with an average of 2 ns.

Using bound relaxation rates and with the average correlation time for dipolar relaxation, proton–Cu(II) distances can be estimated for the various analogues. These are listed in Table II. The large uncertainty in τ_c leads to error limits of ±20% on the distances.

Relaxation of Water Protons. The proton signal of residual water in 99.7 atom % (D) D_2O provides a convenient resonance for T_1 and T_2 measurements by the $T_{1\rho}$ technique. Diamagnetic relaxation rates are minimal for HDO in solution and bound to proteins so that the measured effect of diamine oxidase on $1/T_1$ and $1/T_2$ can be assigned completely to paramagnetic relaxation.

$1/T_2$ at 60, 100, and 220 MHz and $1/T_1$ at 100 and 220 MHz were measured as a function of temperature (Figure 7). Diamine oxidase has a substantial effect on water proton relaxation indicating that water or water protons are exchanging from sites in close proximity to the Cu(II). Estimation of the fraction bound yields values for the bound relaxation rates of the water (Table I), which are found to correspond to a distance of approach of about 3 Å. The temperature dependence in all cases is small, with activation energies less than 1 kcal/mol. However, the uncertainties in line-width measurements at 60 MHz are so great that the slope of this curve could be as low as zero (essentially independent of temperature).

Effects of Deoxygenation. Oxygen is the second substrate of the diamine oxidase reaction and one of the possible roles for Cu(II) would be that of a binding site for O_2. It has been shown by kinetics studies that sufficient O_2 can be removed by simple degassing on a vacuum line to block the aerobic step of the reaction. Mondovi et al. (1967b) showed that addition of substrate to a degassed diamine oxidase solution resulted in the conversion of 1 equiv of substrate to aldehyde without turnover of the enzyme. The concentration of O_2 remaining in solution after degassing must be well below the K_m for O_2.

If O_2 and water compete for a site on Cu(II), the water relaxation should increase after deoxygenation. The experiment was done with a 10 mg/mL solution of diamine oxidase at 100 MHz. It was important to correct for the effects of deoxygenation of the buffer alone in this case. In a metal-free D_2O solution, relaxation of the HDO proton is very inefficient; the presence of a paramagnetic species like O_2 contributes greatly to the relaxation rate. Removal of O_2 from the buffer caused T_2 to change from 8.05 s to 9.63 s and T_1 to change from 14.4 s to 37 s! (This extremely long T_1 is a good check on how metal-free the buffer is.) Relaxation measurements were made on the diamine oxidase solution and then O_2 was removed by several freeze-pump-thaw cycles on the vacuum line. The relaxation measurements were then repeated. Within experimental uncertainty, the enzyme effect on relaxation is independent of the presence of O_2.

HDO Relaxation Rates with Methylhistamine. T_1 and T_2 were measured for residual water protons as a function of added MH I and MH II. The data were corrected for changes in relaxation rates of the buffer HDO by measuring T_1 and T_2 in the concentrated stock solution of the amine assuming linearity with amine concentration between those rates and those of the buffer. Data were also corrected for dilution of the enzyme as inhibitor was added. T_2 of the buffer HDO signal was substantially decreased in the concentrated MH II solution due to chemical exchange effects. In addition, the weak affinity of this amine species required very large concentrations to achieve binding to site 2. At these concentrations the correction for the buffer was larger than the total effect of the enzyme, and the results for T_2 in the presence of MH II are not presented. Figure 8 shows the dependence on MH I concentration of $1/T_1$ and $1/T_2$ for the HDO proton resonance and $1/T_1$ with MH II.

MH I causes a substantial decrease in water relaxation. The effect of MH II is much less pronounced, in line with the different effects of enzyme on the ring proton line widths for the amines themselves. With MH I, the relaxation rates $1/T_1$ and $1/T_2$ are not reduced to zero but instead plateau at high amine concentration. A concentration of about 100 mM MH I is required to produce one-half of the net effect seen on T_1 or T_2. This concentration corresponds approximately to the beginning

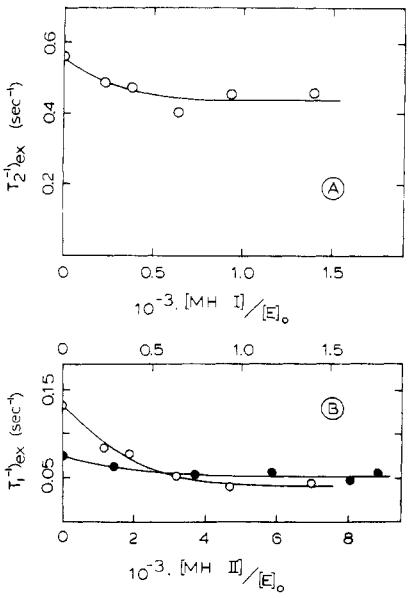


FIGURE 8: MH I and MH II effects on HDO proton relaxation. HDO relaxation rates in the presence of 0.06 mM diamine oxidase are shown vs. the concentration of added methylhistamine. The frequency was 100 MHz, temperature 30 °C. (A) $T_2^{-1}{}_{ex}$ of HDO vs. MH I; (B) (O—O) $T_1^{-1}{}_{ex}$ of HDO vs. MH I; (●—●) $T_1^{-1}{}_{ex}$ of HDO vs. MH II. The curves are corrected for dilution of the enzyme.

of the steep rise in relaxation rates of the H_2 and H_4 ring protons of the amine itself (Figure 5).

Discussion

The results indicate that neither the oxidized end of the substrate molecules nor the product which derives from this portion of the molecule, namely NH_3, binds near the Cu(II). This clearly shows that the original assumptions that the Cu(II) resides near the site of elimination of the amino group and perhaps serves to bind the incoming amino group and correctly orient the substrate molecule for reaction are not valid. In addition, the fact that H_2O binds within an inner sphere ligand distance from the metal ion indicates that this H_2O does not hydrolyze the intermediate imine to generate the aminoaldehyde, which must also occur very near the binding site of the amino group, unless some large conformational change is taking place. Finally, O_2 and H_2O apparently do not compete for a Cu(II) binding site. The Cu(II) therefore apparently is not involved directly in the catalytic process. However, the results with the methylhistamines reveal another process in which the metal ion is involved.

The possibility of there being more than one type of binding site for amines on diamine oxidase is suggested by observations of substrate inhibition with a large number of substrates, particularly histamine. The treatments of this kinetic phenomenon by Haldane (1930) and by Webb (1963) are in terms of a second substrate molecule binding in the active site and preventing reaction of the first. It is this type of binding of a second substrate molecule that our present methylhistamine binding data implicate, and we suggest the possibility that the site which places the imidazole ring in close proximity to the Cu(II) may be the inhibitory binding site. In fact, Zeller (1970) has previously suggested that this may be the function of the Cu(II) in this system. In addition, Werle and Hartung (1956) have found that diethyldithiocarbamate, which effectively chelates with the metal in the enzyme, activates diamine oxidase when histamine is the substrate.

There are, however, several possibilities for a binding mechanism which we cannot distinguish between on the basis of the present data. One possibility is that the two sites we observe are located on the two subunits of the enzyme, in which case the classical views of inhibition by excess substrate are probably not applicable. A second possibility is that there are multiple binding sites on each subunit, with each subunit being able to bind two substrate molecules, the second residing with its imidazole ring very near the metal ion and, by some mechanism, reducing the reactivity. Unfortunately it is not possible to recast this data in terms of Scatchard's equation with sufficient accuracy to determine the numbers of each type of binding site. It is of interest, however, to note the extremely rapid rise in the relaxation effects upon binding of methylhistamine to the second site. Computer analyses of a large variety of binding schemes, including those suggested to explain substrate inhibition, failed to give as rapid an onset of the large relaxation effects as was actually observed. In fact, a general cooperative binding scheme employing one binding site per subunit also failed to give satisfactory agreement. From these analyses we conclude that there are two possible explanations for this phenomenon either of which, if not both, may be applicable. One is that there are in fact more than two total binding sites for methylhistamine on diamine oxidase with a cooperative interaction between the sites. The other possibility is that the bound relaxation rate for methylhistamine bound near the Cu(II) is not a constant quantity. While we do not expect the correlation time, which is related to the properties of the unpaired electron spin on the Cu(II), to change, the bound relaxation rate may be changing due to changes in the distance of approach of the protons to the metal ion. This would imply that the position of one substrate molecule with respect to the Cu(II) is altered upon binding of additional molecules, in which case we have a much more poorly defined binding site. It is clear, however, that there is some type of interaction between the various methylhistamine binding sites.

Finally, the effects of added methylhistamine on the observed water proton relaxation verify that the site occupied at high concentrations places the imidazole ring close enough to the Cu(II) for the ring nitrogen to be a ligand of that metal. It is reasonable to assume that this binding would displace a water ligand from the metal ion. An important feature of the data in Figure 8 is that $1/T_1$ and $1/T_2$ are not reduced to zero, nor are they reduced to one-half, which would be expected if there were two equivalent water ligands, only one of which is displaced upon methylhistamine binding. From the different percentage change of $1/T_1$ and $1/T_2$ (which is more pronounced), we conclude that the nondisplaced water either experiences a substantially different interaction with the Cu(II) or the Cu(II) itself is different in terms of its efficiency in causing relaxation of protons bound nearby. Determination of the exact nature of water proton relaxation effects in this system requires further frequency-dependent measurements and is the subject of another investigation (Kluetz, M. D., and Schmidt, P. G., to be published).

Acknowledgments

We thank Drs. R. E. London and N. A. Matwiyoff for obtaining the ^{15}N spectra and Dr. R. D. White for many helpful suggestions concerning multisite binding.

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Comparative Studies of Oxygen Exchange Catalyzed by Myosin, Heavy Meromyosin, and Subfragment 1. Evidence That the γ-Phosphoryl Group of Adenosine Triphosphate Binds to Myosin in the Region of the (Subfragment 1)–(Subfragment 2) Hinge†

Kamal K. Shukla and Harvey M. Levy*

ABSTRACT: At an intermediate stage in the hydrolysis of Mg^{2+}–ATP by myosin there is an extensive exchange of oxygen between water and the γ-phosphoryl group of the bound nucleotide. The exchange appears to result from a repeated cycle of cleavage and reverse cleavage of the bound ATP, with different oxygens coming into an exchanging position by rotation of the bound phosphate group. Actin activates the overall rate of hydrolysis at a rate-limiting step which follows the exchange reactions; thus, it decreases the time available for exchange when it decreases the turnover time of hydrolysis. By measuring exchange as a function of turnover time, which can be varied by changing the concentration of actin, it is possible to estimate the rate constants for oxygen exchange. Such estimates have indicated that the rate of rotation limits the rate of exchange. With myosin, not only is the average rate of exchange relatively slow, indicating considerable restriction in rotation, but only three of the four oxygens per P_i molecule are able to enter the exchange cycle. Apparently, one of the oxygens is bound to the protein. Comparative studies of oxygen exchange catalyzed by myosin and various single and double-headed fragments of myosin, reported here, suggest that the binding, which limits the extent and rate of oxygen exchange, depends on the integrity of the (S-1)–(S-2) hinge, the flexible region that connects each head of myosin to the body of the molecule. When this hinge is cleaved in the preparation of subfragment 1, then even the fourth oxygen can exchange at a measurable rate, and the average rate constant for exchange of the other three is significantly increased. The results have led us to postulate that one oxygen in the γ-phosphoryl group of ATP, the one that cannot exchange, is bound to myosin in the region of the (S-1)–(S-2) hinge, and that this binding serves to connect points on the flexible hinge to points of the active site that catalyze hydrolysis. An hypothesis is presented that describes how, in this way, changes at the active site, which occur in the course of hydrolysis, could cause the broader movements of the myosin head that occur during contraction.

It has been known for some time that oxygen atoms initially on the γ-phosphoryl group of ATP exchange with oxygens of water at some intermediate stage of Mg^{2+}–ATP hydrolysis by myosin (Levy and Koshland, 1958, 1959; Levy et al., 1960, 1962; Sartorelli et al., 1966). It now appears that this reaction, called intermediate exchange, occurs at step 3 in the following enzymatic pathway (Bagshaw and Trentham, 1974; Bagshaw et al., 1974; Lymn and Taylor, 1971).

$$\mathrm{M} + \mathrm{ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{M{\cdot}ATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{M^{**}{\cdot}ADP{\cdot}P_i}$$

$$\underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ADP{\cdot}P_i} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ADP{\cdot}}$$

$$+ \mathrm{P_i} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} \mathrm{M{\cdot}ADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} \mathrm{M} + \mathrm{ADP}$$

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