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Reactivity of Essential Thiols of Myosin. Chemical Probes of the Activated State†

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ABSTRACT: ^{14}C-Labeled fluorodinitrobenzene and *N*-ethylmaleimide have been used as chemical probes of the conformational states of myosin induced by the binding of MgADP and MgATP. The results indicate that in the high-energy conformation, M**MgADP·P_i, the essential thiols are protected from modification but their diminished reactivity does not result from depletion of the reagent by reaction at nonessential thiols. The binding of MgADP to myosin exposes the essential thiols as reflected by an increased rate of their modification. The influence of the divalent cations Mg^{2+} and Ca^{2+} on the conformation of the M** species has also been investigated. By monitoring the incorporation of fluorodinitrobenzene, the conformations of the M** state in the presence of these cations can be clearly discerned.

A large body of evidence is at hand which demonstrates that the ability of myosin to hydrolyze MgATP is closely linked to the reactivity of two sulfhydryls (designated SH_1 and SH_2) in each of the two globular, subfragment I regions of the molecule (Sekine and Kielley, 1964; Yamaguchi and Sekine, 1966). Recent work from this laboratory has shown that these sulfhydryls exist in close spatial proximity (12–14 Å); they can be bridged by the bifunctional reagent *p*-phenylenedimaleimide (pPDM) (Reisler et al., 1974b). It has also been observed that the near-ultraviolet (UV) circular dichroic (CD) spectrum of myosin covalently bridged between SH_1 and SH_2 by pPDM resembles closely that of myosin in the presence of MgATP, that is in the M**MgADP·P_i conformation (Burke et al., 1976). The M**MgADP·P_i state has been identified with the long-lived, rate-limiting species in the sequence of steps proposed for the binding and hydrolysis of MgATP to myosin (Bagshaw et al., 1974):

$$\mathrm{M} + \mathrm{MgATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{M{\cdot}MgATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{M^*MgATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{M^{**}MgADP{\cdot}P_i} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{M^*MgADP{\cdot}P_i} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{M^*{\cdot}MgADP} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{M{\cdot}MgADP} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} \mathrm{M} + \mathrm{MgADP}$$

The involvement of SH_1 and SH_2 in the interaction with the substrate is also suggested by the observation that in the M**MgADP · P_i conformation these thiols are not readily reacted with sulfhydryl reagents, whereas in the M*MgADP conformation they are both exposed to such reagents (Schaub et al., 1975). The decreased reactivity of the essential sulfhydryls in the M** state was attributed by us to a protective effect imposed by the bound nucleotide (Reisler et al., 1974a). In fact, the quantitative measurement of the protective effect was used as a chemical assay of the M** state and was shown to monitor the shift in the relative population of this state in response to changes in temperature or ionic strength of the solvent (Harrington et al., 1975).

Recently, Schaub and Watterson and their collaborators (Schaub et al., 1975; Watterson et al., 1975) have suggested from their studies of incorporation of [^{14}C]MalNEt[1] that the decreased reactivity of the SH_1 and SH_2 thiols in the M**MgADP · P_i state results from a depletion of the reagent through reaction with a class of nonessential thiols exposed by the binding of the nucleotide. This conclusion is not consistent with our interpretation and with our postulate on a direct interaction between the sulfhydryls and the substrate in the M**MgADP·P_i conformation. Since the properties of the M** complex and the state of the essential sulfhydryls in this complex are central to our understanding of the mechanism of ATP hydrolysis we have reexamined the effect of the nu-

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[1] Abbreviations used are: MalNEt, *N*-ethylmaleimide; N_2phF, 1-fluoro-2,4-dinitrobenzene (FDNB is used in the figures); EDTA, ethylenediaminetetraacetic acid.

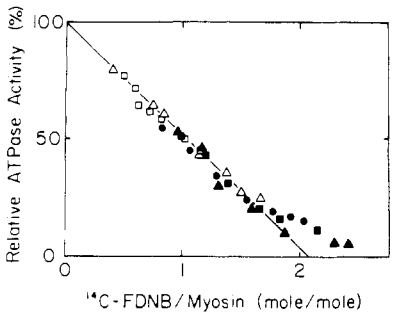

FIGURE 1: The relative loss in EDTA ATPase activity as a function of incorporation of [^{14}C]N_2phF by myosin reacted in the presence of no ligand (●), MgADP (▲), MgATP (△), CaADP (■), and CaATP (□). Myosin (1-2 mg/mL) in 0.5 M KCl-0.05 M Tris-HCl (pH 7.9) was reacted with an eightfold molar excess of [^{14}C]N_2phF at 5 °C. The concentration of ligand was 10^{-2} M in all cases.

cleotide induced conformations M**MgADP·P_i and M*MgADP on the reactivity of SH_1 and SH_2 groups. Employing ^{14}C-labeled N_2phF and MalNEt we have been able to demonstrate that the M**MgADP·P_i conformation existing at high ionic strength and low temperature does protect the SH_1 and SH_2 groups from modification and their decreased reactivity is not a consequence of the exposure of a class of nonessential sulfhydryls as suggested by Schaub et al. (1975). However, in agreement with these workers and in contrast to our earlier conclusions (Reisler et al., 1974a), we find that the binding of MgADP to myosin, producing the M*MgADP conformation, causes an exposure of these sulfhydryls and an increase in their reactivity. Additionally, incorporation studies with [^{14}C]N_2phF demonstrate that the M** state formed with CaATP protects the essential sulfhydryls to a greater extent than the analogous state formed with MgATP. This reflects an altered conformation of the M** state which is dependent on the nature of the divalent cation.

Materials and Methods

Glass distilled water was used throughout. Experiments were done at 5 °C unless otherwise stated. [^{14}C]MalNEt (sp act. 7-25 mCi/mmol) and [^{14}C]N_2phF (sp act. 17 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.) and Amersham (Radiochemical Center). ATP and ADP were the products of Sigma (St. Louis, Mo.). All other reagents were analytical grade. Myosin was prepared by the procedure of Godfrey and Harrington (1970). Reaction of myosin with MalNEt and N_2phF was carried out as described elsewhere (Reisler et al., 1974a). [^{14}C]MalNEt was used undiluted with carrier and its concentration was determined by absorbance (E_{305nm} = 620); in the case of [^{14}C]N_2phF the reagent was diluted 68-fold with carrier and the specific activity was remeasured by the method of Shaltiel and Soria (1969). The conditions of high ionic strength and low temperature were chosen since they favor the formation of the M** state as the predominant species in the presence of the substrate (Harrington et al., 1975). High concentrations of the ligands (10^{-2} M) and low protein concentrations (1-2 mg/mL) were employed to ensure that less than 20% of the substrate was hydrolyzed during the course of the incorporation reaction. With [^{14}C]N_2phF the reaction was terminated by precipitation of the myosin with 20 vol of cold distilled water. This procedure is necessary since cysteinyl *S*-dinitrophenyl derivatives are unstable in the presence of 2-mercaptoethanol and dithiothreitol (Shaltiel, 1967). The precipitated protein was collected by centrifugation dissolved in 0.5 M KCl, and reprecipitated by addition of water. This procedure was repeated twice. The protein was subsequently dissolved in a buffer system containing 0.5 M KCl and 5×10^{-3} M Tris-HCl at pH 7.9 and dialyzed exhaustively against this solvent. In the case of [^{14}C]MalNEt the reaction was terminated by addition of dithiothreitol to a concentration of 2×10^{-3} M and the proteins were then dialyzed in the same solvent, as described above for N_2phF-treated myosin, to remove non-protein-bound [^{14}C]MalNEt. Aliquots (200 μL) of protein solution (~1.5×10^{-10} mol of myosin) plus 3.0 mL of Aquasol were loaded into miniscintillation vials for counting. ATPase activities were determined by the procedure of Kielley and Bradley (1956) and Kielley et al. (1956). Protein concentrations were determined by absorption ($A_{280\,nm}^{1\%}$ = 5.5) for unmodified myosin and by the microbiuret procedure for the modified samples.

TABLE 1: Relative ATPase Activities of Modified Myosins.

System	Reagent	Conditions[a]	Rel ATPase act.[b] CaATPase	Rel ATPase act.[b] EDTA ATPase
Myosin			100	100
Myosin	N_2phF	4 (−)	405	20
SH_1-N_2ph myosin	N_2phF	8 (+)	420	10
SH_1-N_2ph myosin	MalNEt	8 (+)	40	0
SH_1-MalNEt myosin			250	10
SH_1-MalNEt myosin	N_2phF	4 (+)	230	3
SH_1-MalNEt myosin	MalNEt	4 (+)	10	0

[a] The values in this column indicate the molar excess of reagent over myosin employed in the reaction. The signs in parentheses signify whether the reaction was done in the presence (+) or absence (−) of 10^{-3} M MgADP. [b] 100% ATPase activities correspond to 1 and 3 μmol of P_i mg^{-1} min^{-1} myosin for the Ca^{2+} and EDTA ATPases, respectively.

Results

Incorporation Studies with [^{14}C]N_2*phF.* N_2phF has certain advantages over MalNEt as a chemical probe of conformational perturbations in myosin. Bailin and Bárány (1972) have established by peptide isolation and characterization that the reagent has a high specificity for modification of the SH_1 site. Moreover, as evidenced by data presented in Table I, the reagent is apparently unable to react at the SH_2 site even under conditions which favor rapid modification by MalNEt (10^{-3} M MgADP, pH 7.9). Thus, the reagent labels the SH_1 site and is insensitive to conformational changes around the SH_2 thiol. The observation that modification of essential thiols of myosin by N_2phF is confined to the SH_1 site alone immensely simplifies the correlations between the modification reaction and the changes in the activity properties of the protein.

The incorporation of [^{14}C]N_2phF and its effect on the EDTA ATPase activity of myosin reacted in the M**MgADP·P_i, M*MgADP, and unliganded states are shown in Figure 1. It is clear that in all three cases there is a constant linear relationship between the loss of EDTA ATPase and the amount of label incorporated by the protein. Extrapolation of these data to the abscissa shows that the EDTA ATPase is abolished when 2 mol of the reagent is incorporated per mol of myosin. At the same time the CaATPase activity is activated indicating that in each case the reaction is directed specifically to the SH_1 site with little modification occurring at nonessential thiols. The invariant specificity of N_2phF toward SH_1 groups under different reaction conditions (Figure

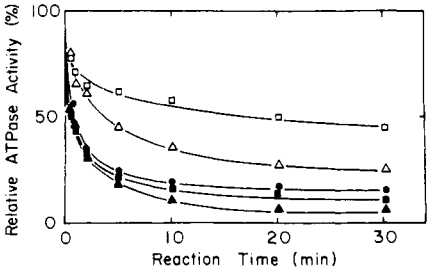

FIGURE 2: Relative EDTA ATPase activity of myosin as a function of reaction time with [^{14}C]N_2phF. Conditions and symbols as in Figure 1.

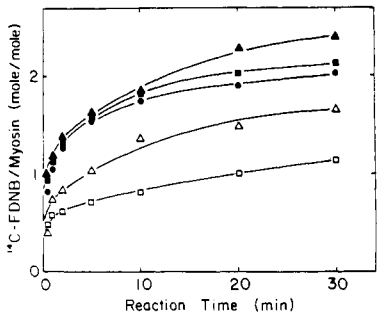

FIGURE 3: Rate of incorporation of [^{14}C]N_2phF by myosin reacted in the presence of 10^{-2} M ligands. Reaction conditions and symbols are as in Figure 1.

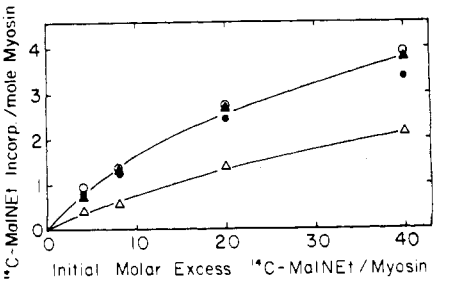

FIGURE 4: Influence of ligands on incorporation of [^{14}C]MalNEt by myosin. Myosin (1-2 mg/mL) in 0.5 M KCl-0.05 M Tris-HCl (pH 7.9) was reacted for 3 min at 5 °C with the molar excess of [^{14}C]MalNEt indicated on the abscissa in the presence of no ligand (●), $MgCl_2$ (○), MgADP (▲), and MgATP (△).

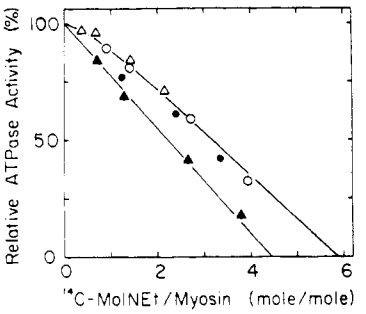

FIGURE 5: Relative EDTA ATPase activity of myosin as a function of [^{14}C]MalNEt incorporation. Conditions and symbols are as in Figure 4.

1) is particularly advantageous. It simplifies and reduces the analysis of the effect of nucleotide binding on the reactivity of the SH_1 thiol to examination of changes in the EDTA ATPase activity. These changes are linear with the incorporation of the reagent at the SH_1 site in the various conformations of myosin-nucleotide complexes.

In Figure 2 we have examined the reactivity of the SH_1 thiol by measuring the rate of change of the EDTA ATPase during modification of the protein in the presence and absence of MgADP and MgATP. Clearly, in the presence of MgATP, when the protein is predominantly in the M** state, the low rate of the loss of EDTA ATPase demonstrates a marked decrease in the reactivity of the SH_1 thiol. On the other hand, the binding of MgADP to the protein produces a conformation in which the SH_1 group is somewhat more reactive than in the unliganded system. The incorporation of the label under these reaction conditions is shown in Figure 3. Because of the invariant specificity of the reagent (under different reaction conditions) for the SH_1 site and its apparent inability to react with other sulfhydryls under the conditions chosen, the present data demonstrate that the SH_1 is protected from modification in the M** state and it is exposed to reaction in the M*MgADP conformation.

Incorporation Studies with MalNEt. Since the data obtained with N_2phF clearly demonstrated that the SH_1 thiol was protected in the M** state, it was of interest to determine whether a similar conclusion could be reached by using MalNEt as a conformational probe. We have previously observed that in the M** state existing in high salt and low temperature the rate of attack of the essential thiols by MalNEt is reduced (Reisler et al., 1974a). This result can be interpreted as (1) reflecting a protected environment of the essential thiols in this conformation (Reisler et al., 1974a) or (2) stemming from a depletion of the pool of MalNEt by reaction at a class of reactive, nonessential sulfhydryls exposed in the M** configuration (Schaub et al., 1975).

The incorporation of [^{14}C]MalNEt into myosin in the presence and absence of $MgCl_2$, MgADP, and MgATP as a function of the molar ratio of MalNEt to myosin is shown in Figure 4. It is evident that the binding of $MgCl_2$ and MgADP has little effect on the extent of incorporation in comparison to the unliganded system. On the other hand, the steady-state conformation M**MgADP·P_i formed in the presence of MgATP produces a significant decrease in the extent of incorporation of the label reflecting a decreased reactivity of myosin thiols in this conformation.

The analysis of the accompanying EDTA ATPase activity data is somewhat more complex in the case of MalNEt since the reagent has the ability to label both the SH_1 and SH_2 thiols in the presence of nucleotides. Inactivation of the EDTA ATPase activity reflects modification at either one or both groups and can be used as a measure of the degree of modification of these thiols.

The results of such analyses are presented in Figure 5. For any level of incorporation of MalNEt by the protein the greatest loss of EDTA ATPase occurs for myosin reacted in the M*ADP conformation. Thus, although the M*MgADP and the unliganded conformations differ but little in the total incorporation of MalNEt (Figure 4) the extents of modification at the essential thiols are markedly different. Clearly, modification of the M*MgADP conformation is more specific toward the essential thiols. In contrast to this, the low rate of EDTA ATPase loss found on modification in the M** state results from a protecting effect of MgATP as evidenced by a decreased level of labeling. Thus, under the experimental conditions employed in this study, the reactivity in the M** state cannot be attributed to reactions at a class of nonessential thiols as suggested by Schaub et al. (1975).

*Chemical Probing of the Influence of Divalent Cation on the M** State.* So far we have presented evidence on the MgATP induced protection of the essential thiols of myosin against chemical modification. This protective effect, or rather

TABLE II: Influence of MgATP and CaATP on Extent of [^{14}C]MalNEt Incorporation of Myosin and Its Effect on EDTA ATPase Activity.

[^{14}C]MalNEt initial molar excess over myosin[a]	Ligand present in system			
	MgATP		CaATP	
	Incorp.[b]	Rel act.[c]	Incorp.[b]	Rel act.[c]
0	0	100	0	100
4	0.40	97	0.36	96
8	0.66	96	0.57	93
20	1.39	84	1.30	79
40	2.15	71	2.08	63

[a] Reaction was carried out for 5 min at 5 °C. For other details see Materials and Methods. [b] Incorporation moles of [^{14}C]MalNEt/mol of myosin. [c] Relative activity is EDTA ATPase activity. For unmodified myosin 100% activity corresponds to 3 μmol of P_i mg^{-1} min^{-1}.

its quantitation, provides a chemical assay of the M** state. The accepted test for this conformation, enhancement of the intrinsic fluorescence of myosin, does not distinguish between myosin conformations induced by reaction with CaATP or MgATP (Werber et al., 1972). A recent report based on chemical probes suggests that the M** states formed with either divalent cation may be identical (Watterson et al., 1975). Yet, the presumed identity of conformation is neither reflected in the stability of the two complexes nor in their functional role. We have reexamined this question by employing both [^{14}C]-MalNEt and [^{14}C]N_2phF as chemical probes of these conformations.

The incorporation of MalNEt and its influence on the EDTA ATPase of myosin in these two states are shown in Table II. No significant difference could be detected in the reaction of MalNEt with the CaATP and MgATP induced M** state. However, when N_2phF was employed, a clear distinction between the two M** states was obtained. The results shown in Figures 2 and 3 attest that the presence of Ca^{2+} renders the SH_1 thiol less reactive than in the presence of the Mg^{2+} complex.

Discussion

The reasons behind the choice of N_2phF as our test reagent become apparent upon examination of the results presented in Table I and Figure 1. The reaction with N_2phF is directed only toward one of the two essential sulfhydryls (SH_1) and its specificity for that group is not affected by the presence of nucleotides in the reaction mixture. In other words, a single value of EDTA ATPase corresponds to a given level of incorporation of N_2phF under all the reaction conditions tested. This relationship is sustained despite changes in the conformation of the protein induced by binding of MgADP, CaADP, CaATP, and MgATP. In all cases N_2phF modification causes marked increases in the CaATPase activity of myosin signifying modification of the SH_1 thiols in each myosin head. Such a pattern of myosin modification allows a straightforward interpretation and detection of nucleotide effect on the course of the reaction.

The results presented in Figure 2 demonstrate that the reactivity of the SH_1 site of myosins in different conformations can be presented in the increasing order of M** < M < M*. The same conclusion can be derived from the data shown in Figure 3, where a markedly lower rate of incorporation of [^{14}C]N_2phF is exhibited by the M** state. The combined data obtained with N_2phF show that the essential thiol SH_1 is protected from modification in the M** conformation. The binding of MgADP, on the other hand, does not protect the SH_1 thiol and in fact exposes this group for reaction as suggested by Schaub and his collaborators (1975).

In contrast to the high and uniform specificity exhibited by N_2phF for the SH_1 thiol (under different reaction conditions), MalNEt reacts both at the SH_1 (Sekine and Kielley, 1964) and SH_2 sites (Yamaguchi and Sekine, 1966; Schaub et al., 1975). The incorporation data presented in Figure 4 show similar uptake of [^{14}C]MalNEt in the M*MgADP and unliganded conformations and reduced incorporation in the M**MgADP·P_i state. Yet, despite similar levels of incorporation exhibited by the M*MgADP and unliganded conformations the sites of modification in these two cases are different. Extrapolation of the data in Figure 5 to the abscissa yields the amounts of incorporated [^{14}C]MalNEt required to abolish the EDTA ATPase of myosin reacted in the various conformations. This analysis leads to values of 4.4 and 6 mol of reagent per mol of myosin in the M*MgADP and unliganded conformations. In the M**MgADP·P_i state 6 mol of MalNEt is required for the same goal. Thus, modification at nonessential thiols is occurring both in the unliganded and the M**MgADP·P_i conformations. However, in the M** state the overall activity of essential and nonessential thiols is reduced (Figure 4) indicating protection of SH_1 and SH_2 groups from modification by MalNEt.

The data presented in this contribution show that the conformational transition in myosin accompanying the kinetic step M**MgADP·P_i $\rightleftharpoons$ M*MgADP + P_i is paralleled by transition of the essential thiols from a protected environment to a more exposed one. Such a transition is inherent in the recently proposed model of ATP hydrolysis by myosin involving direct interaction between MgATP and the essential thiol groups (Reisler et al., 1974a). Thus, our data support the validity of the chemical assay of the activated state.

In view of the low affinity of thiols to complex with Mg^{2+}, it is unlikely that the postulated interaction occurs directly through the divalent metal although such a possibility cannot be ruled out on this basis alone. The recent observation of Bagshaw and Reed (1976) concerning the relative positions of the divalent metal complexes of ADP and the spin-labeled SH_1 thiol cannot be readily extrapolated to the unmodified M** state since the modification itself precludes the formation of such a state (Harrington et al., 1975). Moreover, it seems likely that the spin labeling perturbs the relative position of MgADP and SH_1 on the protein. It has been observed, for example, that binding of MgADP to unmodified protein causes a shift in the spacing of SH_1 and SH_2 groups of at least 5 Å, bringing them to a closer proximity (Burke and Reisler, 1977).

If the formation of the M** conformation is a prerequisite for muscle contraction it remains unclear why CaATP cannot cause contraction of glycerinated muscle fibers. Transient kinetic studies by Lymn and Taylor (1970) have shown that the rates of binding CaATP and of cleavage of the terminal phosphoryl group by myosin are identical with those obtained with MgATP. Furthermore, the much faster steady-state rate observed with CaATP is apparently due to a faster decay of the steady-state complex. Physical (Werber et al., 1972) and chemical (Watterson et al., 1975) studies failed to detect any difference between the CaATP and MgATP complexes with myosin. This, of course, cannot be taken to signify that such complexes are identical. Indeed, when N_2phF is used as the chemical probe, the SH_1 site is protected to a significantly different extent by MgATP and CaATP. This result, which reveals structural differences in the complexes of myosin with CaATP and MgATP, is in line with crystallographic evidence

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†

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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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