Magnetic Resonance Studies of the Conformations of Enzyme-Bound Substrates

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Measurement of nuclear magnetic relaxation rates in the presence of paramagnetic probes, which determines distances from individual atoms to a nearby paramagnetic reference point, has emerged in the past decade as a useful approach to the study of the conformation and arrangement of enzyme-bound substrates.[1-5] Such NMR studies in solution, like x-ray diffraction in the crystalline state, detect individual atoms. The obvious advantage of observing complexes in solution, with directly measurable kinetic and thermodynamic properties, is that the relevance of such complexes to catalysis is testable.

The NMR method most thoroughly tested by comparison of some 14 distances determined in solution[2-4] with the corresponding crystallographic distances is the measurement of paramagnetic effects on the longitudinal relaxation rates ($1/T_1$) of substrate nuclei at several magnetic fields. Distances ≤23 Å from a paramagnetic center have been calculated[3] with precisions of ±10%, and conformations of enzyme-bound substrates with molecular weights as large as 824 (propionyl-coenzyme A)[5] have been determined. Limitations of the method include the limited range of accurately measurable distances, the complexity of the analysis when more than one paramagnetic species is present,[5] and a requirement for fast exchange between free and bound substrates.

A comprehensive review of this method has been written.[6] Although it was originally worked out to study enzyme–substrate interactions, the method is, in principle, applicable to a wide variety of molecular interactions in biochemistry, such as the binding of haptens and antigens with antibodies, the conformations of drugs and hormones when bound to their receptors, and the interactions of ligands with the lipid, protein, and carbohydrate components of membranes.

This Account considers the applications of the paramagnetic probe—$1/T_1$—method to determination of the conformations of flexible substrates when bound to enzymes. The results are relevant to the mechanisms of two classes of enzyme reactions: (a) carbonyl polarization and (b) phosphoryl- and nucleotidyl-transferring reactions.

Methodology

Since the development of the theory of paramagnetic effects on nuclear relaxation rates[7] and its first application to metal–substrate interactions on enzymes,[1] several reviews[1,6] and textbooks[8] have treated the techniques for measuring relaxation rates and the quantitative interpretation of such data. We therefore provide here only a qualitative summary of the experimental approach.

The longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of a population of magnetic nuclei represent first-order rate constants for equilibration of the magnetization of the spins along the magnetic field, and for dephasing of the spins in a plane perpendicular to the magnetic field, respectively. Pulsed NMR methods have long been used for measuring the relaxation rates of solvent protons,[9] and continuous-wave NMR methods have been used to measure the relaxation rates of fluorine,[1] protons,[1] and phosphorus atoms[3-5,10,11] of enzyme-bound substrates. The introduction of Fourier transform methods[12] has greatly facilitated $1/T_1$ measurements[10-14] and has also permitted relaxation studies of the ^{13}C nucleus.[13]

We shall give attention to the paramagnetic effect of an unpaired electron, for example, an enzyme-bound transition metal or spin label, on the longitudinal relaxation rate ($1/T_{1p}$) of a nearby magnetic nucleus. The

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(1) A. S. Mildvan, M. Cohn, and J. S. Leigh, Jr., in "Magnetic Resonance in Biological Systems", A. Ehrenberg, B. G. Malmstrom, and T. Vanngard, Ed., Pergamon Press, New York, N.Y., 1967, p 113; A. S. Mildvan, J. S. Leigh, Jr., and M. Cohn, *Biochemistry*, **6**, 1805 (1967); A. S. Mildvan and M. C. Scrutton, *ibid.*, **6**, 2978 (1967); A. S. Mildvan and M. Cohn, *Adv. Enzymol.*, **33**, 1 (1970); A. S. Mildvan and J. L. Engle, *Methods Enzymol.*, **26C**, 654 (1972); M. Cohn and J. Reuben, *Acc. Chem. Res.*, **4**, 214 (1971); A. Lanir and G. Navon, *Biochemistry*, **11**, 3536 (1972); A. C. McLaughlin, J. S. Leigh, and M. Cohn, *J. Biol. Chem.*, **251**, 2777 (1976).

(2) A. S. Mildvan, T. Nowak, and C. H. Fung, *Ann. N.Y. Acad. Sci.*, **222**, 192 (1973).

(3) R. K. Gupta, *J. Biol. Chem.*, **251**, 6815 (1976).

(4) D. L. Sloan and A. S. Mildvan, *Biochemistry*, **13**, 1711 (1974).

(5) C. H. Fung, R. J. Feldmann, and A. S. Mildvan, *Biochemistry*, **15**, 75 (1976).

(6) A. S. Mildvan and R. K. Gupta, *Methods Enzymol.*, in press.

(7) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962); Z. Luz and S. Meiboom, *ibid.*, **40**, 2686 (1964); I. Solomon, *Phys. Rev.*, **99**, 559 (1955); I. Solomon and N. Bleombergen, *J. Chem. Phys.*, **25**, 261 (1956); N. Bloembergen and L. O Morgan, *ibid.*, **34**, 842 (1961); A. Lanir, S. Gradstajn, and G. Navon, *Biochemistry*, **14**, 242 (1975); S. H. Koenig, R. D. Brown, and J. Studebaker, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 551 (1971).

(8) J. W. Emsley, J. Feeney, and L. H. Sutcliffe in "High Resolution NMR Spectroscopy", Vol. 1, Pergamon Press, New York, N.Y., 1965, p 520; T. L. James in "NMR in Biochemistry", Academic Press, New York, N.Y., 1975, p 177; R. A. Dwek in "NMR in Biochemistry", Clarendon Press, Oxford, 1973, p 174.

(9) M. Cohn and J. S. Leigh, *Nature (London)*, **193**, 1037 (1962); M. Cohn, *Biochemistry*, **2**, 623 (1963).

(10) T. Nowak, A. S. Mildvan, and G. L. Kenyon, *Biochemistry*, **12**, 1690 (1973).

(11) T. Nowak and A. S. Mildvan, *Biochemistry*, **11**, 2819 (1972).

(12) R. R. Ernst and W. A. Anderson, *Rev. Sci. Instrum.*, **37**, 93 (1966); R. L. Vold, J. S. Waugh, M. P. Klein, and D. E. Phelps, *J. Chem. Phys.*, **48**, 3831 (1968).

(13) C. H. Fung, A. S. Mildvan, A. Allerhand, R. Komoroski, and M. C. Scrutton, *Biochemistry*, **12**, 620 (1973).

(14) G. G. McDonald and J. S. Leigh, Jr., *J. Magn. Reson.*, **3**, 358 (1973).

nearby nucleus might be, for example, a proton or phosphorus atom of a substrate which is exchanging into the paramagnetic enzyme complex. From theory[3,7,8] the paramagnetic effect depends predominantly on four parameters: the lifetime of the complex (τ_m), the relative stoichiometry or coordination number (q) of the substrate and the paramagnet in the complex, the correlation time for electron–nuclear dipolar interaction (τ_c), and the distance (r) from the unpaired electron to the nucleus in the complex. The paramagnetic effect on the transverse relaxation rate ($1/T_{2p}$) of this nucleus is a different function of these same four parameters and also contains contact and chemical shift contributions resulting from contact and dipolar interactions. The paramagnetic contribution to the transverse relaxation rate ($1/T_{2p}$) sets a lower limit to $1/\tau_m$, the pseudo-first-order rate constant for dissociation of the substrate from the paramagnetic complex,[11] and measurements of $1/T_{2p}$ as a function of temperature may be used, in suitable cases, to evaluate $1/\tau_m$ and provide kinetic information on the observed complex.[1,15] When $1/T_{2p}$ exceeds $1/T_{1p}$ by an order of magnitude or more it may safely be concluded that the rate constant for dissociation of the substrate ($1/\tau_m$) greatly exceeds $1/T_{1p}$, i.e., that the lifetime of the complex (τ_m) contributes little to T_{1p}, eliminating one of the four unknown parameters in $1/T_{1p}$.[11]

The correlation time (τ_c) can be evaluated by measurements of $1/fT_{1p}$ at several magnetic fields, appropriately taking into account that τ_c is itself dependent on the magnetic field[2-7] and that zero-field splitting may contribute to the relaxation rates at low magnetic fields.[6,7] The two remaining parameters, q and r, cannot be separately evaluated by measurements of $1/T_{1p}$ alone. However, an independent evaluation of the stoichiometry (q) of the paramagnet and substrate in the complex by appropriate binding studies permits a direct calculation of the distance (r). Since $1/T_{1p}$ is inversely proportional to the sixth power of r, errors in the measurement of $1/T_{1p}$ and in the evaluation of τ_c are minimized and in consequence errors in the determination of r are at most 10%.

Conversely, an independent evaluation of the metal to water proton distance r by x-ray analysis of model complexes permits direct calculation of q,[10,16] the coordination number for rapidly exchanging water ligands on an enzyme-bound metal, and permits estimation of changes in q as substrates bind to the enzyme–metal complex. In this type of calculation there is no minimization of errors, and uncertainties in estimates of q may be as great as 25–50%. When binary metal–substrate as well as ternary enzyme–metal–substrate complexes are present, the paramagnetic effects in the ternary complex are calculated from a study of the binary complex, and from the calculated composition of the system, with use of all of the relevant dissociation constants, determined independently.

When a set of distances from a paramagnetic center to several nuclei of a bound substrate have been determined from $1/T_{1p}$, the conformation of the substrate in the paramagnetic complex is determined by model building.[10,11] This often requires a systematic computer search among thousands of conformations to eliminate those which require significant van der Waals overlap or which require distances to the paramagnetic reference point that exceed the values measured experimentally.[5,17,18]

Since most biochemical reactions involve the simultaneous interaction of two or more substrates with an enzyme, intersubstrate distances may be needed as well as substrate conformations to clarify an enzyme mechanism. For such measurements a paramagnetic analogue of one of the substrates is required;[19] its effects on the longitudinal relaxation rates of nuclei of the other substrate on the enzyme are measured and analyzed as outlined above. The first measurements of intersubstrate distances were made on dehydrogenases using a spin-labeled analogue of NAD.[19] More recently, Tempo–ATP,[20] a paramagnetic analog of ATP, Cr^{3+}–ATP, a substitution-inert paramagnetic analog of MgATP,[21] and R-CoA, a paramagnetic ester of coenzyme A,[22,23] have been used to determine intersubstrate distances on other enzymes.

Applications of Magnetic Resonance Methods

Carbonyl Polarizing Enzymes. Transcarboxylase, which has been purified and characterized by Wood and co-workers,[24] catalyzes the transfer of a carboxyl group from methylmalonyl-CoA to pyruvate by way of an enzyme-bound biotin. The enzyme has been found by EPR and atomic absorption analyses to contain Cu^{2+} in addition to Zn^{2+} and Co^{2+}.[25] The total metal content is ~12 mol/mol or ~2 metal ions/biotin. From $1/T_{1p}$ of water protons at various frequencies, two rapidly exchanging water ligands on the enzyme-bound Co^{2+} were detected, while the Cu^{2+} was inaccessible to water. Formation of the enzyme–pyruvate complex decreased the number of fast-exchanging water ligands on Co^{2+} by 1. However, the Co^{2+} to pyruvate distances, as calculated from $1/T_{1p}$ of the carbon atoms and protons of pyruvate by ^{13}C and proton NMR (5.0–6.3 Å), indicated a second-sphere complex (Figure 1)[25]. Hence pyruvate binding in the second coordination sphere appears to have immobilized an inner-sphere water ligand so that it no longer exchanges rapidly. These results suggest that the metal-bound water ligand might polarize the carbonyl group of pyruvate by hydrogen

(15) M. C. Scrutton and A. S. Mildvan, *Arch. Biochem. Biophys.*, **140**, 131 (1970).
(16) J. Reuben and M. Cohn, *J. Biol. Chem.*, **245**, 6539 (1970).
(17) C. D. Barry, J. Glasel, R. J. P. Williams, and A. V. Xavier, *J. Mol. Biol.*, **84**, 471 (1974); C. D. Barry, D. R. Martin, R. J. P. Williams, and A. V. Xavier, *ibid.*, **84**, 491 (1974); B. Furie, J. H. Griffen, R. J. Feldmann, A. Sokoloski, and A. N. Schechter, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 2344 (1974).
(18) D. L. Sloan, L. A. Loeb, A. S. Mildvan, and R. J. Feldmann, *J. Biol. Chem.*, **250**, 8913 (1975).
(19) H. Weiner, *Biochemistry*, **8**, 526 (1969); A. S. Mildvan and H. Weiner, *J. Biol. Chem.*, **244**, 2465 (1969); A. S. Mildvan and H. Weiner, *Biochemistry*, **8**, 552 (1969); A. S. Mildvan, L. Waber, J. J. Villafranca, and H. Weiner in "Structure and Function of Oxidation Reduction Enzymes", A. Åkeson and Å. Ehrenberg, Ed., Pergamon Press, New York, N.Y., 1972, p 745.
(20) T. R. Krugh, *Biochemistry*, **10**, 2594 (1971).
(21) R. K. Gupta, C. H. Fung, and A. S. Mildvan, *J. Biol. Chem.*, **251**, 2421 (1976).
(22) S. W. Weidmann, G. R. Drysdale, and A. S. Mildvan, *Biochemistry*, **12**, 1874 (1973).
(23) C. H. Fung, R. K. Gupta, and A. S. Mildvan, *Biochemistry*, **15**, 85 (1976).
(24) H. G. Wood, H. Lochmuller, C. Reipertinger, and F. Lynen, *Biochem. Z.*, **237**, 247 (1963); D. B. Northrop and H. G. Wood, *J. Biol. Chem.*, **244**, 5820 (1969); F. Ahmad, B. Jacobson, B. Chuang, W. Brattin, and H. G. Wood, *Biochemistry*, **14**, 1606 (1975).
(25) C. H. Fung, A. S. Mildvan, and J. S. Leigh, Jr., *Biochemistry*, **13**, 1160 (1974).

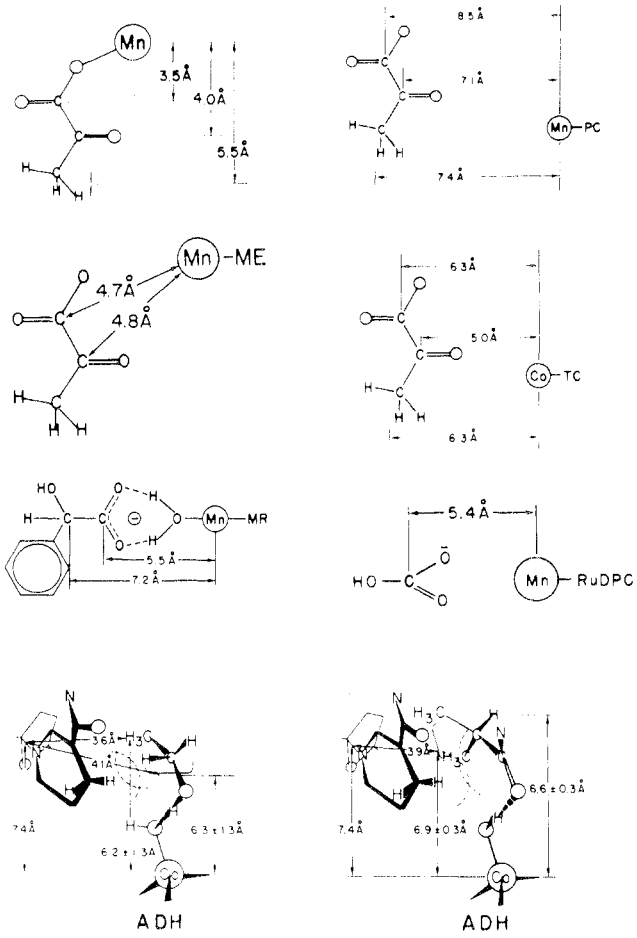

Figure 1. Comparison of binary Mn^{2+}–pyruvate complex with conformations of six second-sphere complexes in solution as determined by nuclear relaxation: ADH, alcohol dehydrogenase;[28] TC, transcarboxylase;[25] PC, pyruvate carboxylase;[13] RuDPC, ribulose diphosphate carboxylase;[27] ME, malic enzyme;[26] MR, mandelate racemase.[29]

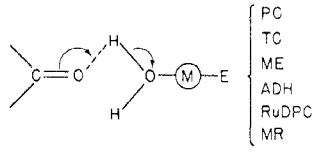

Figure 2. General mechanism of carbonyl polarization by enzyme-bound metal ions based on the data of Figure 1.

bonding or protonation (Figure 2). Similar results were obtained with the Mn^{2+} metalloenzyme pyruvate carboxylase[13] which catalyzes the same half-reaction as transcarboxylase (Figure 1). In the binary Mn–pyruvate complex in solution, monodentate carboxyl coordination is observed (Figure 1).[13] Hence the active sites of these enzymes alter the metal–substrate interaction, preventing direct coordination.

Second-sphere complexes are also detected on four other enzymes which use divalent cations to polarize carbonyl groups of their substrates: malic enzyme, which activates pyruvate,[26] ribulose diphosphate carboxylase,[27] which activates CO_2, alcohol dehydrogenase,[28] which activates aldehydes, and mandelate racemase, which catalyzes the interconversion of D- and L-mandelate[29] (Figure 1).

From these six examples (Figures 1 and 2), it appears that a general mode of activating carbonyl groups of substrates by enzyme-bound metals is not by direct coordination but rather by protonation via an intervening water ligand (Figure 2). The participation of a metal-bound water in the alcohol dehydrogenase reaction is further supported by the detection of a small inverse secondary kinetic solvent isotope effect,[30] in accord with a model study of a known deprotonation of a metal-bound water ligand.[31]

The conformation of the other enzyme-bound substrate of transcarboxylase, propionyl-CoA[5], and its distance from enzyme-bound pyruvate[23] were also studied by measurements of $1/T_{1p}$. Distances from the bound Co^{2+} at the active site to seven protons (6.5–8.7 Å) as well as lower limit distances to five additional protons (≥7.4 Å) and to the three phosphorus atoms (≥9.0 Å) of bound propionyl-CoA were used in a computer search among 47 000 rotamers for that conformation of propionyl-CoA which provided the best fit to the distances and minimized van der Waals overlaps. The best fit structure shows a partially unfolded U shape about the Co^{2+} with an anti adenine–ribose conformation (Figure 3A).[5] The intersubstrate distance between pyruvate and propionyl-CoA was determined using a spin-labeled thioester of CoA (Figure 3B) which binds at the CoA ester site of the enzyme.[23] These distances indicate that pyruvate and propionyl-CoA are bound near the same metal ion on transcarboxylase, as shown in Figure 3A.[23]

From a composite model of the bound substrates on transcarboxylase, it is concluded that carboxybiotin moves, at most, only ~7 Å in transferring CO_2 from methylmalonyl-CoA to pyruvate during catalysis (Figure 3C);[23] this was unexpected since biotin is on a 14-Å long arm when extended and the two half-reactions of transcarboxylase occur on different subunits. The role of this long arm thus appears to be to place the transferred carboxyl group at the end of a long probe, permitting it to traverse the gap which occurs at the interface of three subunits and to be located between the CoA and pyruvate sites (Figure 3D).[23] Independent evidence for the proximity of the two substrates of transcarboxylase has recently been obtained by Rose et al., who detected a small amount of enzyme-catalyzed tritium transfer between pyruvate and propionyl-CoA.[32]

A concerted mechanism consistent with the geometric data, with steric considerations, and with the isotopic studies has been proposed.[23,32]

Phosphoryl- and Nucleotidyl-Transfer Enzymes. *Muscle Pyruvate Kinase.* This enzyme, the first studied by $1/T_1$ measurements of substrates,[1,2] catalyzes reversible phosphoryl-transfer reactions between P-enolpyruvate and ADP, as well as irreversible phosphoryl transfer from ATP to F^-, NH_2OH, and glycolate. Nuclear relaxation studies of four active enzyme–Mn–substrate complexes by ^{13}C, ^{31}P, and ^1H NMR[13,33-36]

(26) R. Y. Hsu, A. S. Mildvan, G. G. Chang, and C. H. Fung, *J. Biol. Chem.*, **251**, 6574 (1976).
(27) H. Miziorko and A. S. Mildvan, *J. Biol. Chem.*, **249**, 2743 (1974).
(28) D. L. Sloan, J. M. Young, and A. S. Mildvan, *Biochemistry*, **14**, 1998 (1975).
(29) E. T. Maggio, G. L. Kenyon, A. S. Mildvan, and G. D. Hegeman, *Biochemistry*, **14**, 1131 (1975).
(30) J. P. Klinman, Sixth Steenbock Symposium on Isotope Effects on Enzyme Catalyzed Reactions, in press.
(31) R. K. Gupta and A. S. Mildvan, *J. Biol. Chem.*, **250**, 246 (1975).
(32) I. A. Rose, E. L. O'Connell, and F. Solomon, *J. Biol. Chem.*, **251**, 902 (1976).

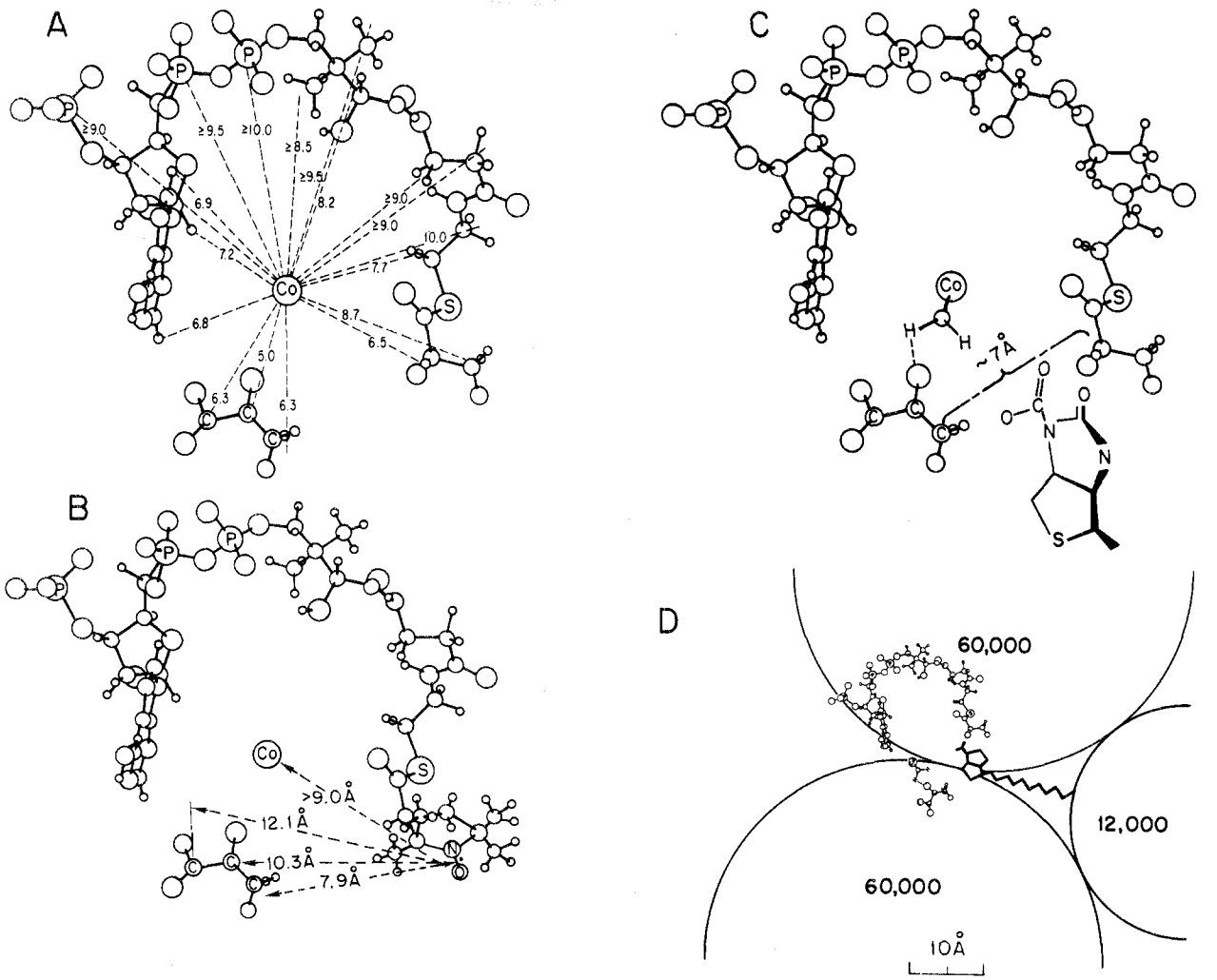

Figure 3. Structure of transcarboxylase complexes. (A) Conformation of propionyl-CoA and pyruvate on transcarboxylase determined by computer using the indicated distances (angstrom units).[5] (B) Intersubstrate distances from R-CoA to pyruvate and to bound Co^{2+} on transcarboxylase.[23] (C) Arrangement and conformations of pyruvate, propionyl-CoA, and carboxybiotin. (D) Diagram to scale of transcarboxylase subunits,[24] showing the role of the 14-Å arm of biotin.[23]

revealed only second-sphere complexes of the transferable phosphoryl group (Figure 4). The nucleotide conformation in the pyruvate kinase–Mn–ATP complex differs greatly from that in the binary Mn–ATP complex,[34] emphasizing the danger of using distances in a binary complex to predict those on an enzyme (Figure 4).

Cr^{3+}–ATP, a substitution-inert metal–ATP complex, has been shown to be active in promoting the enolization of pyruvate in the presence of pyruvate kinase and an enzyme-bound divalent cation.[21] In addition to the enzyme-bound divalent cation, the nucleotide bound cation has been shown by kinetic studies to be essential for catalysis of the enolization of pyruvate.[36] The intersubstrate distance on pyruvate kinase has been determined by the paramagnetic effects of CrATP on the relaxation rates of the $^{13}C_1$, $^{13}C_2$, and the methyl protons of pyruvate in the active complex (Figure 4).[21] A composite model of the enzyme-bound substrates based on 15 distances in four active complexes of pyruvate kinase (Figure 5)[37] revealed molecular contact between the phosphorus of the γ-phosphoryl group of ATP and the carbonyl oxygen of pyruvate, consistent with direct phosphoryl transfer. Hence the NMR results argue against but do not strictly exclude phosphoenzyme or metaphosphate intermediates.[37] Crystallographic studies of cat muscle pyruvate kinase and its binary substrate complexes at 6-Å resolution[38] indicate proximity of the binding sites for the enzyme-bound divalent cation, P-enolpyruvate, and ATP in accord with the magnetic resonance data.

Second-sphere distances from the enzyme-bound Mn^{2+} to phosphorus atoms of interacting ligands are also found by $1/T_1$ methods in the phosphoryl-transferring enzymes phosphoglucomutase[39] and the (Na^+ + K^+)-activated ATPase,[40] although only inactive substrate analogues were used in these cases. Similarly, a predominantly (≥85%) second-sphere complex has been detected between the enzyme-bound Mn^{2+} at the active site of DNA polymerase I and the reaction-center α-phosphorus atom of dTTP,[18] suggesting that sec-

(33) E. Melamud and A. S. Mildvan, *J. Biol. Chem.*, **250**, 8193 (1975).
(34) D. L. Sloan and A. S. Mildvan, *J. Biol. Chem.*, **251**, 2412 (1976).
(35) T. James and M. Cohn, *J. Biol. Chem.*, **245**, 3519 (1974).
(36) R. K. Gupta, R. M. Oesterling, and A. S. Mildvan, *Biochemistry*, **15**, 2881 (1976).
(37) A. S. Mildvan, D. L. Sloan, C. H. Fung, R. K. Gupta, and E. Melamud, *J. Biol. Chem.*, **251**, 2431 (1976).
(38) D. K. Stammers and H. Muirhead, *J. Mol. Biol.*, **95**, 213 (1975).
(39) W. J. Ray and A. S. Mildvan, *Biochemistry*, **12**, 3733 (1973).
(40) C. M. Grisham and A. S. Mildvan, *J. Supramol. Struct.*, **3**, 304 (1975).

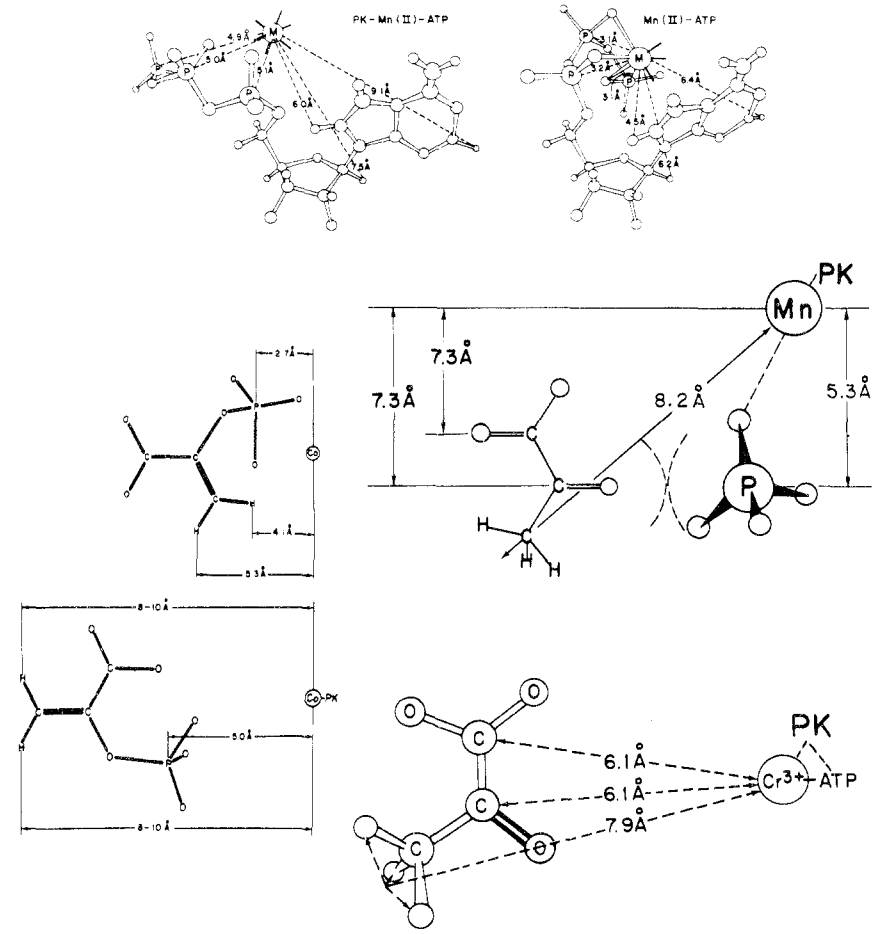

Figure 4. Comparison of conformations in solution of active complexes of pyruvate kinase[13,21,33-36] with those of the binary Mn–ATP complex[34] and of the binary Co–P-enolpyruvate complex.[33]

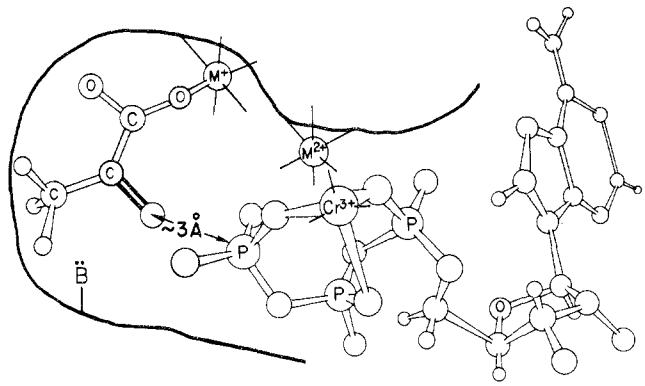

Figure 5. Arrangement and conformations of substrates at the active site of pyruvate kinase based on the data of Figure 4.[37]

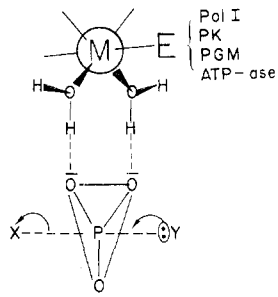

Figure 6. General mechanism of phosphoryl activation based on distances on the enzyme DNA polymerase, Pol I;[18] pyruvate kinase, PK;[33,34] phosphoglucomutase, PGM;[39] and the (Na^{+} + K^{+})ATPase.[40]

ond-sphere complexes may be a general mode of activating phosphoryl groups for nucleophilic attack (Figure 6).[37]

DNA Polymerase. DNA polymerase I from *E. coli* catalyzes the in vitro synthesis of DNA.[41] Using four deoxynucleoside triphosphate substrates, this enzyme accurately copies a DNA template, elongating the DNA primer chain according to the Watson–Crick base-pairing scheme, making less than one mistake in 10^5 nucleotides. Each chain elongation step results from the nucleophilic displacement of pyrophosphate on the substrate by the 3′-OH group of the preceding sugar of the growing chain. DNA polymerases are Zn metalloenzymes,[42] and the enzyme-bound Zn appears to interact with the DNA template–primer complex.[42] These enzymes also require a divalent cation such as Mg^{2+} or Mn^{2+} for activity which binds tightly to the enzyme.[43] Using Mn^{2+} at this site as a paramagnetic probe, we have mapped the conformation of the substrates dTTP and dATP by ^{31}P and proton relaxation rates (Figure 7A,B).[18]

(41) T. Kornberg and A. Kornberg, *Enzymes, 3rd Ed.*, **10**, 119 (1974); A. Kornberg, "DNA Synthesis", W. H. Freeman, San Francisco, Calif., 1974; L. A. Loeb, *Enzymes, 3rd Ed.*, **10**, 173 (1974).

(42) J. P. Slater, A. S. Mildvan, and L. A. Loeb, *Biochem. Biophys. Res. Commun.*, **44**, 37 (1971); C. F. Springgate, A. S. Mildvan, R. Abramson, J. L. Engle, and L. A. Loeb, *J. Biol. Chem.*, **248**, 5987 (1973); B. J. Poiesz, N. Battula, and L. A. Loeb, *Biochem. Biophys. Res. Commun.*, **56**, 959 (1974); B. J. Poiesz, G. Seal, and L. A. Loeb, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4892 (1975).

(43) J. P. Slater, I. Tamir, L. A. Loeb, and A. S. Mildvan, *J. Biol. Chem.*, **247**, 6784 (1972).

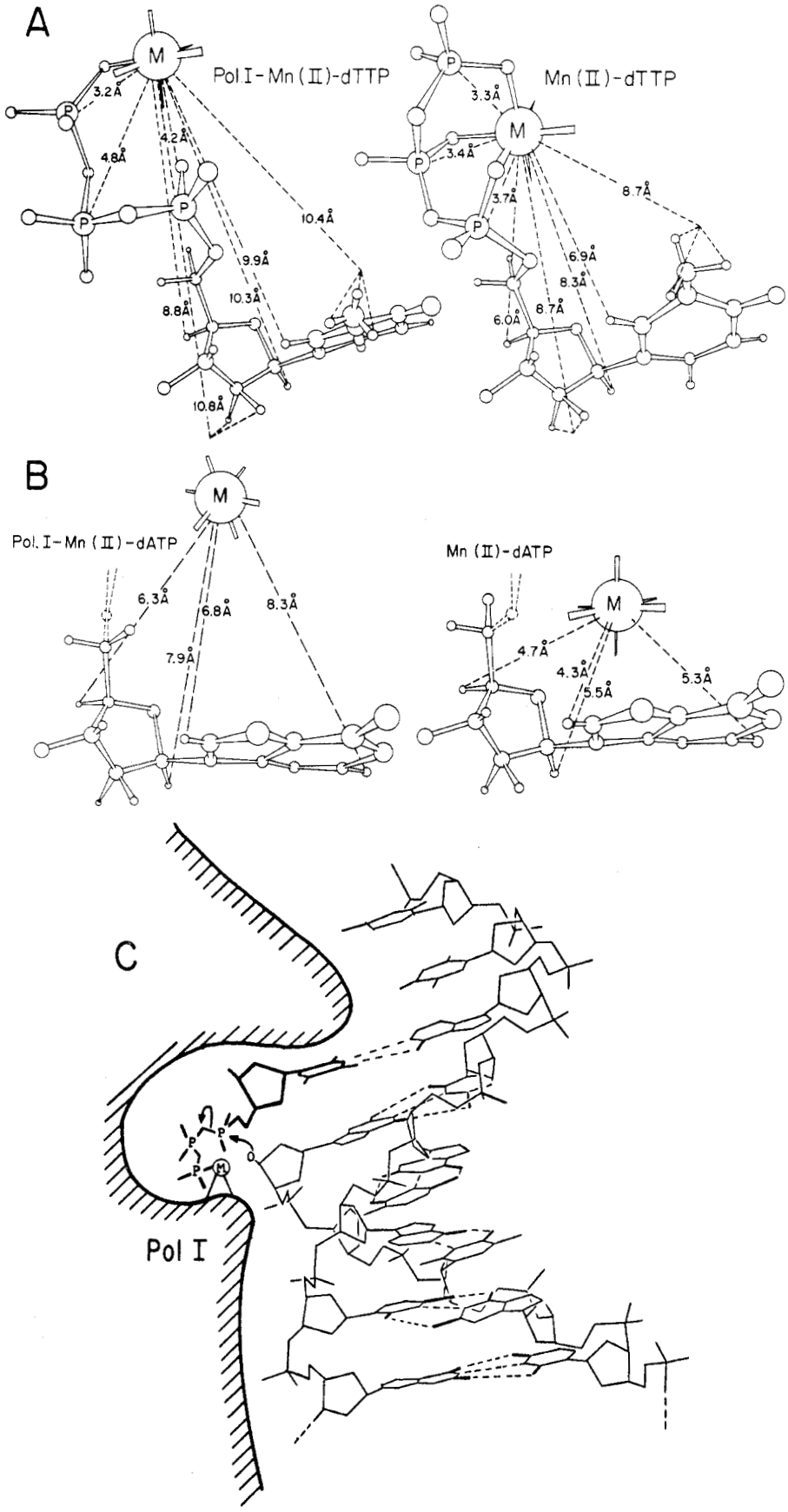

Figure 7. Conformations and distances in DNA polymerase complexes in solution.[18] (A) Binary Mn–dTTP and ternary DNA polymerase–Mn–dTTP complexes. (B) Binary Mn–dATP and ternary DNA polymerase–Mn–dATP complexes. (C) Mechanism of chain elongation catalyzed by DNA polymerase consistent with the structures.

Two differences are noted between the binary and ternary complexes. First, in the binary complex all three of the phosphoryl groups of dTTP are directly coordinated to Mn^{2+}. On DNA polymerase only the

γ-phosphoryl group remains coordinated by the enzyme-bound Mn^{2+}. The distance to the reaction-center α-phosphorus atom (4.2 Å) is most simply explained by the rapid averaging of ≤15% inner-sphere coordination with ≥85% second-sphere coordination. The resulting polyphosphate conformation is puckered and somewhat strained. Hence an important role of the divalent cation activator in catalysis is to assist the departure of the leaving pyrophosphate group by γ coordination, and possibly to facilitate nucleophilic attack on the α-phosphorus atom by strain and by hydrogen bonding through a coordinated water ligand.[18]

A second difference between the binary and ternary complexes is in the conformational angle χ about the thymine–deoxyribose bond of dTTP. The χ value of 40 ± 5° in the binary complex increases to 90 ± 5° in the ternary complex (Figure 7A). Similarly, a 90° torsion angle is also found for the purine nucleotide substrate Mn–dATP when bound to DNA polymerase (Figure 7B).[18] Interestingly, the latter torsion angle of 90° is that found for the deoxynucleotidyl units in double-helical DNA. Hence the binding of the substrate Mn–dTTP to the enzyme, DNA polymerase, in the absence of template, has changed the substrate conformation to that of a nucleotidyl unit in the product—double-helical DNA.

When the structure of enzyme-bound Mn–dTTP is superimposed by computer onto the double-helical structure of DNA-B (Figure 7C), the resulting location of the α-phosphorus atom and the leaving pyrophosphate group of the bound substrate relative to the attacking 3′-OH group of the preceding nucleotide unit is consistent only with an in-line nucleophilic displacement on the α phosphorus.[18] Hence the biosynthesis of nucleic acids, like their hydrolysis,[44] appears to proceed by an in-line mechanism.

(44) D. A. Usher, E. S. Erenrich, and F. Eckstein, *Proc. Nat. Acad. Sci. U.S.A.*, **69**, 115 (1972).

The selection by the enzyme of those substrate conformations that fit into the double helix would amplify the Watson–Crick base-pairing scheme and would explain the low error rates of DNA polymerases[41] which are at least two orders of magnitude below those predicted by the thermodynamic[45] and kinetic effects of base-pairing alone.[46]

Conclusions

Numerous examples have been provided which establish that the average conformation of a flexible substrate, when bound to an enzyme, generally differs from that of the free substrate in solution (Figures 1, 4, and 7).

Second-sphere enzyme–metal–(H_2O)–substrate complexes are used by enzymes to polarize carbonyl groups (six examples, Figures 1 and 2) and to position phosphoryl groups for nucleophilic attack (four examples, Figures 4–7). In the case of pyruvate kinase, an additional metal interacts directly with the ATP (Figure 5).

On two-substrate enzymes, such as dehydrogenases (Figure 1), kinases (Figure 5), and even on a biotin enzyme, (Figure 3), close proximity of the two bound substrates is observed, in some cases approaching molecular contact.

The inner coordination sphere of a metal is used to facilitate the departure of the leaving group in DNA polymerase (Figure 7).

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(45) A. S. Mildvan, *Annu. Rev. Biochem.*, **43**, 357 (1974).
(46) E. C. Travaglini, A. S. Mildvan, and L. A. Loeb, *J. Biol. Chem.*, **250**, 8647 (1975).

Structural Effects on the Acid-Catalyzed Hydration of Alkenes

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Acid-catalyzed hydration of olefins, a relatively late entry in the field of mechanistic investigations, was first studied in the early 1930's. At the same time, the anti-Markownikoff addition of HBr to alkenes, which was soon formulated as a free-radical chain process, was

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Scheme I

$$\text{olefin} + H_3O^+ \rightleftharpoons \left[\begin{array}{c} H \\ | \\ C{=}C \end{array}\right]^+ + H_2O$$

1

$$1 + H_2O \overset{\text{slow}}{\rightleftharpoons} R^+ \ldots OH_2$$

Scheme II

$$H_3O^+ + \text{olefin} \overset{\text{slow}}{\rightleftharpoons} R^+ + H_2O$$

being elucidated.[1] In 1934 Lucas and his co-workers published[2] measurements of the rate constants for