Reaction of Essential Lysyl Residues of Pig Heart Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase with 2,4-Pentanedione†

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ABSTRACT: The rate of inactivation of pig heart DPN-specific isocitrate dehydrogenase by 2,4-pentanedione is pseudo-first-order and linearly dependent on reagent concentration. Isocitrate in combination with manganous ion can prevent inactivation, and a dissociation constant (K_{IC}) for the enzyme-isocitrate complex can be calculated which is similar in magnitude to the K_m for isocitrate under the same conditions. Although neither the cofactor, DPN, nor the allosteric activator, ADP, prevents inactivation by reagent, ADP lowers both K_{IC} and K_m to the same extent. These data suggest that the reagent may be reacting with residues within a binding site for manganous-isocitrate. DPNH accelerates the inactivation and also enhances protection by isocitrate, lowering K_{IC} by a factor of 20. Because ADP does not prevent the DPNH rate enhancement, it is unlikely that the two nucleotides compete for identical binding sites. Reaction with 2,4-pentanedione thus provides a probe of the mode of ligand interaction with the enzyme. Inactivation appears to result from the reaction of 2,4-pentanedione with lysyl residues to form enamines. The occurrence of a new absorbance band during inactivation and the isolation by gel filtration of enzyme with an absorbance peak at 312 nm are consistent with enamine formation. Hydroxylamine, which abolishes the 312-nm peak, also causes appreciable reactivation of the enzyme. By use of [2,4-^{14}C]-2,4-pentanedione, it was established that reaction of an average of no more than 3 lysines of the 26 per peptide chain resulted in complete inactivation; and an average of only 2 lysines react when enzymatic activity is retained in the presence of 50 mM isocitrate. Reaction with arginine was excluded by the unchanged amino acid composition of modified enzyme. These data suggest that formation of an enamine of possibly 1, and certainly no more than 3, lysine residue(s) in the catalytic center of the enzyme is responsible for inactivation by 2,4-pentanedione.

A continuing series of investigations in this laboratory has been directed at the identification of residues that are involved in the active site of pig heart DPN-dependent isocitrate dehydrogenase (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) by studying inactivation as a result of specific covalent modification. This work has the additional objective of exploiting the effects of substrates, cofactors, and activators on the rate of inactivation as a probe of the normal binding sites of the enzyme. By the use of a variety of reagents, several residues have thus far been implicated in a catalytic or substrate binding site of this enzyme: a lysyl residue is carbamoylated by reaction with cyanate (Shen and Colman, 1975), carboxyl groups which are essential to catalysis are modified by a water-soluble carbodiimide (Ramachandran and Colman, 1975), and a cysteine which is carboxymethylated by iodoacetate appears to be involved in a site for manganous-isocitrate which is important for maintenance of protein stability (Mauck and Colman, 1976).

The present studies use 2,4-pentanedione, a compound similar in size to the substrate, isocitrate, both as a modifier and as a probe of the effect on binding of isocitrate caused by the presence of either the allosteric modifier, ADP, or the reduced cofactor, DPNH. It is concluded that the reagent reacts with a lysyl residue in the catalytic center of the enzyme which is involved in binding the substrate, manganous-isocitrate. The binding of manganous-isocitrate, as ascertained from protection against inactivation, is dramatically enhanced by the presence of DPNH, and this effect appears distinct from a similar enhancement caused by ADP.

Materials and Methods

Materials. The reagent, 2,4-pentanedione, was either Fisher Reagent Grade (redistilled, bp 129–133 °C) or Aldrich Gold Label (99+%). Radioactive pentanedione ([2,4-^{14}C]-2,4-pentanedione) was synthesized by California Bionuclear Corp. and had a specific activity of 0.5 μCi/μmol. Pipes,[1] cellulose phosphate, ADP, DPN, DPNH, and sodium DL-isocitrate were purchased from Sigma Chemical Co., DEAE-cellulose (DE-52) was from Reeves-Angel, and Sephadex G-25 was from Pharmacia. All other chemicals were reagent grade.

Enzyme Preparations. The DPN-dependent isocitrate dehydrogenase was prepared from pig hearts by modification of the method of Shen et al. (1974). Enzyme, homogeneous by the criterion of electrophoresis on polyacrylamide gels containing 2% sodium dodecyl sulfate (Shen et al., 1974), was prepared from fresh hearts obtained from a local abattoir. After ammonium sulfate precipitation, the preparation was chromatographed on DEAE-cellulose and cellulose phosphate. Most of the reported experiments were conducted with homogeneous enzyme. In some cases preliminary studies were carried out with enzyme from the DEAE-cellulose fractionation. Because the susceptibility to inactivation by 2,4-pentanedione is somewhat dependent upon the purity of an enzyme preparation, this information is specified where necessary.

Enzyme Activity, Protein, and Amino Acid Determinations. Enzyme activity was determined spectrophotometrically at 340

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received August 17, 1976.* This investigation was supported by grants from the United States Public Health Service (2-R01-AM 17552) and the Delaware Heart Association.

[1] Abbreviations used: Pipes, piperazine-*N*-*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid.

nm in a Gilford spectrophotometer, using a recorder at 0.1 absorbance full-scale, by adding a small aliquot (2–5 μL) to 1 mL of a standard assay solution (Cohen and Colman, 1972) containing 1.0 mM DPN, 1.33 mM $MnSO_4$, 20 mM DL-isocitrate, and Tris–acetate buffer (33 mM in acetate, pH 7.2). For specific activity determinations the cell compartment was maintained at 25 °C, but in inactivation experiments the circulating water was usually 30 °C. Protein concentration was estimated by multiplying the absorbance measured at 280 nm in a cuvette of 1 cm light path by the factor 1.55 mg/mL (Shen et al., 1974). An enzyme unit is defined as the reduction of 1 μmol of DPN per min at 25 °C, and specific activity is in units per mg of protein. A molecular weight of 40 000 for the enzyme subunit was used in all calculations (Shen et al., 1974). For the determination of amino acid composition the enzyme was dialyzed against 50 mM NH_4HCO_3 buffer and dried repeatedly in vacuo in order to remove the volatile buffer. The enzyme sample was hydrolyzed in 6 N HCl, as described previously (Shen et al., 1974), and measurements were made on a Beckman 120 amino acid analyzer using an expanded scale.

Inactivation and Spectral Studies. For studies of the kinetics of inactivation an incubation solution was prepared which had a final composition of 50 mM sodium Pipes, pH 6.2, 2 mM $MnSO_4$, 20% glycerol, and 0.44 M KCl (μ = 0.5 M). To this solution was added a suitable aliquot of a freshly diluted 1 M aqueous solution of 2,4-pentanedione, and then the reaction was initiated by the immediate addition of the enzyme sample. In experiments where isocitrate was added, KCl of equivalent ionic strength was omitted. No other additions to the incubation mixture caused a significant alteration in ionic strength. The DPNH solutions were freshly prepared, and their concentrations were estimated from their absorbance at 340 nm. The protein concentration was usually from 0.1 to 0.2 mg per mL. Small aliquots were removed from the tightly stoppered incubation tubes at intervals for activity determination in the standard assay. Except where indicated, inactivation experiments and other kinetic determinations were performed at 30 °C, and rates of inactivation were corrected for the spontaneous decay of an enzyme sample incubated with all additions but reagent.

The change in absorbance at 325 nm of the inactivation solution was monitored in a thermostated 1-mL cuvette in either a Gilford spectrophotometer or a Cary Model 15 spectrophotometer. Because the actual temperature in the cell compartment differed from that of the circulating water, the cuvette temperature was determined by means of a Telethermometer equipped with a fine probe (Yellow Springs Instrument Co.). Many of the experiments where loss of activity was correlated with an increase in absorbance at 325 nm were carried out at 15 °C in order to avoid the slight haziness that resulted from protein denaturation at 30 °C. Activity determinations were made on small aliquots taken from the incubation cuvette.

Procedure with Radioactive 2,4-Pentanedione. The [2,4-^{14}C]-2,4-pentanedione was diluted with an approximately equal volume of nonradioactive 2,4-pentanedione before use. The concentration of reagent and specific radioactivity in an incubation mixture were estimated from the absorbance at 275 nm ($\epsilon_{275} = 2.30 \times 10^4$ M^{-1} cm^{-1}) and the radioactivity of a 1000-fold dilution of the reaction mixture in which the reagent was employed. The radioactivity was measured in a Packard Tri-Carb scintillation counter, Model 3300, using Aquasol solutions.

Determination of Michaelis and Inhibition Constants in the Presence of Reagent. The Michaelis constants for isocitrate and DPN, as well as the inhibition constant for DPNH, were determined under conditions similar to those used in the incubation mixtures of enzyme and 2,4-pentanedione. The concentration of each substrate was varied in turn, with the remaining substrates being maintained constant at 20, 2, and 1 mM for DL-isocitrate, $MnSO_4$, and DPN, respectively. The concentrations of 2,4-pentanedione, Pipes, and KCl were 0.1 M, 50 mM, and 0.44 M, respectively, and 20% glycerol was present. When 2,4-pentanedione was added to a Pipes buffer solution, there was a rapid increase in absorbance at 340 nm. Although the cause of this increase is unknown, it is complete within 5–10 min, and its extent is apparently small, as ascertained from the insignificant loss of the characteristic absorbance of 2,4-pentanedione at 278 nm. Accordingly, the reagent was preincubated with the buffer solution for at least 30 min before use in kinetic measurements involving isocitrate dehydrogenase. When the K_m of DPN or the K_I of DPNH was being determined, enzyme was preincubated in the reaction mixture which lacked only DPN for about 30 s prior to the initiation of the reaction by the addition of a variable concentration of DPN. The 20 mM isocitrate in these solutions protected the enzyme against reagent during the preincubation period. However, when the K_m for isocitrate, with or without ADP, was under investigation, the enzyme was added last to the assay solution which contained all the components of an inactivation solution plus 1 mM DPN. Enzyme concentrations for these determinations were about 1 μg per mL.

Determination of the Association Constant for Mn^{2+} with 2,4-Pentanedione. The published formation constant at 30 °C for the complex between the 2,4-pentanedione anion and Mn^{2+} is 1.51×10^4 M^{-1}, and the ionization constant for 2,4-pentanedione is 1.12×10^{-9} M (Izatt et al., 1954). Because the concentration of free Mn^{2+} may control the mechanism by which isocitrate and metal interact with the enzyme (Cohen and Colman, 1974), the effective binding constant of Mn–2,4-pentanedione was measured under conditions similar to those in the incubation solutions. Pipes buffer, 10 mM, pH 6.0, was made 485 μM in 2,4-pentanedione and scanned against buffer alone in a Cary Model 15 spectrophotometer. The peak shifted from 278 nm to nearly 285 nm at saturating Mn^{2+}. The greatest increase in absorbance occurred at 300 nm ($\Delta\epsilon = 1.4 \times 10^3$ M^{-1} cm^{-1}) and this wavelength was selected for titration. An association constant at pH 6 of 185 M^{-1} was determined, and this value was used in calculations of the concentrations of free Mn^{2+} and the various species of isocitrate by use of the computer program described by Cohen and Colman (1972) with the substitution of 2,4-pentanedione for chloride.

Results

Inactivation by 2,4-Pentanedione. Isocitrate dehydrogenase can be totally inactivated by treatment for several hours with 0.1 M 2,4-pentanedione at pH 6.0 and 30 °C. The loss of activity follows pseudo-first-order kinetics for at least the first 75% of the reaction, as shown in Figure 1, line A. Marked protection is afforded by 20 mM DL-isocitrate in the presence of manganous ion and ADP (Figure 1, line B). A plot of the pseudo-first-order rate constant (k_i) as a function of 2,4-pentanedione concentration is linear, yielding a second-order rate constant of 2.8×10^{-1} min^{-1} M^{-1}, when the enzyme used is homogeneous. The rate of inactivation is somewhat dependent upon the purity of the enzyme, with the k_i values for crude enzyme (specific activity of 2) being as much as 50% lower than those for homogeneous enzyme (specific activity of at least 30).

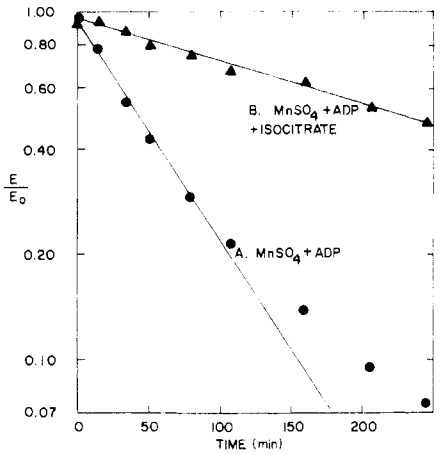


FIGURE 1: Inactivation of DPN-dependent isocitrate dehydrogenase by 0.1 M 2,4-pentanedione at 30 °C in Pipes buffer, pH 6.2. The detailed conditions are described in the Materials and Methods section. The enzyme was purified by chromatography on DEAE-cellulose and had a specific activity of 2 units/mg. The experimental enzyme activities were corrected for the loss of activity of a control sample incubated in the same solutions except for reagent. (A) $MnSO_4$ (2 mM) and ADP (2 mM) were present in reaction mixture. (B) $MnSO_4$ (2 mM), ADP (2 mM), and DL-isocitrate (20 mM) were present in reaction mixture. The pseudo-first-order rate constants are 14.7×10^{-3} and 2.78×10^{-3} min^{-1} for lines A and B, respectively.

TABLE I: Effect of Ligands on the Rate of Inactivation of DPN-Dependent Isocitrate Dehydrogenase by 2,4-Pentanedione.[a]

	Additions to Reaction Mixture[b]			
	$MnSO_4$ (mM)	ADP (mM)	Other	$k_{i(obsd)}$ ($min^{-1} \times 10^3$)
1	0	2	0	41
2	0	2	50 mM DL-isocitrate	26
3	2	0	0	14
4	2	2	0	14
5	2	2	16 mM DPN	13
6	2	2	50 mM DL-isocitrate	1.2

[a] The inactivation procedure is described in the Materials and Methods section. The enzyme used for this set of experiments was purified by chromatography on DEAE-cellulose and had a specific activity of 1-2 units/mg. The reagent was 0.1 M, the incubation temperature 30 °C, and the pH of the reaction mixtures 6.15. [b] When $MnSO_4$ was not included in the incubation solution, 3 mM EDTA was added to chelate the $MnSO_4$ contributed by the enzyme solution.

Table I records the effect of ligands on the pseudo-first-order rate constant for inactivation by 0.1 M 2,4-pentanedione. A comparison of lines 1 and 4 indicates that the addition of the activating metal, 2 mM Mn^{2+}, causes about a threefold decrease in the rate of inactivation. In a separate experiment it was found that increasing the Mn^{2+} concentration to 10 mM further reduced k_i by only 30%. The rate is not further reduced when either the allosteric activator, ADP, or the coenzyme, DPN, is added together with the 2 mM metal ion (lines 3-5), indicating that these nucleotides do not themselves influence the rate of inactivation. Isocitrate in the absence of Mn^{2+} has little effect (line 2 vs. line 1). The most striking decrease in the rate of inactivation occurs when isocitrate is added together with $MnSO_4$ and ADP (lines 6 vs. 4), suggesting that reaction occurs within a substrate binding site.

TABLE II: Effect of Isocitrate with and without ADP on the Rate of Inactivation of DPN-Dependent Isocitrate Dehydrogenase by 2,4-Pentanedione.

DL-Isocitrate Concentration (mM)	$k_{i(obsd)}$[a] (10^3 min^{-1})		K_{IC} (mM)	
	−ADP	+ADP[b]	−ADP	+ADP
2.5	21.7	19.2	13.2	7.3
5.0	15.9	9.85	8.0	3.1
25	10.8	5.35	18.0	6.1

[a] Enzyme was incubated with 0.1 M 2,4-pentanedione at 30 °C in the presence of 2 mM $MnSO_4$, as described in the Materials and Methods section. The rate in the absence of either isocitrate or ADP was 25.8×10^{-3} min^{-1}. The enzyme was a homogeneous sample (specific activity >30) purified by DEAE-cellulose and cellulose phosphate chromatography. [b] The ADP concentrations were either 2 or 5 mM; the effects were indistinguishable.

The rate of inactivation is decreased progressively by increasing concentrations of isocitrate in the presence of metal ion, as shown in Table II. Furthermore, ADP does enhance the protection produced by the substrate, as indicated by a somewhat lower rate constant in the presence of the allosteric activator at each concentration of isocitrate. Dissociation constants for the enzyme–isocitrate complex were calculated from the data of Table II by means of eq 1:

$$k_{i(obsd)} = \frac{k_i}{1 + (IC)/K_{IC}} \quad (1)$$

where $k_{i(obsd)}$ is the rate constant in the presence of isocitrate, k_i the rate constant with 2 mM Mn^{2+}, (IC) the concentration of DL-isocitrate, and K_{IC} the apparent dissociation constant for enzyme–total isocitrate complex (Shen and Colman, 1975). Typical dissociation constants, which are listed in Table II, give an average of 13 mM in the absence and 6 mM in the presence of 2 mM ADP.

If the inactivation results from modification of a residue in the active center, a resemblance between the dissociation constant for enzyme–substrate complex and its Michaelis constant under the same conditions would be expected. The kinetically determined K_m values for isocitrate in the 2,4-pentanedione incubation medium were 3.2, without, and 1.6 mM, with 2 mM ADP. These are of the same order of magnitude as the dissociation constants given in Table II; it is not known at present what factors account for the observed differences between the K_m and K_{IC} values, although one factor may be the markedly higher protein concentrations used in the inactivation experiments as compared with the K_m determinations. The values of the Michaelis constant are much higher than those previously reported for pH 6 (Cohen and Colman, 1972). If 2,4-pentanedione is deleted from the assay solution, the K_m values are reduced only slightly, to 2.0 mM without ADP and 0.7 mM with ADP. The relatively high concentration of KCl seems to be responsible for the elevated values which are observed for the Michaelis constant since, if KCl is omitted, the values drop dramatically to 34 μM without, and 12 μM, with, 2 mM ADP. The magnitude of the allosteric activation by ADP seems to be diminished in Pipes buffer from the tenfold reduction in the K_m for isocitrate observed in imidazole buffer (Cohen and Colman, 1972). Nevertheless, ADP does lower the K_{IC} calculated from the protection against inactivation by 2,4-pentanedione (Table II) to the same degree as it decreases the K_m for isocitrate, and the similarity between

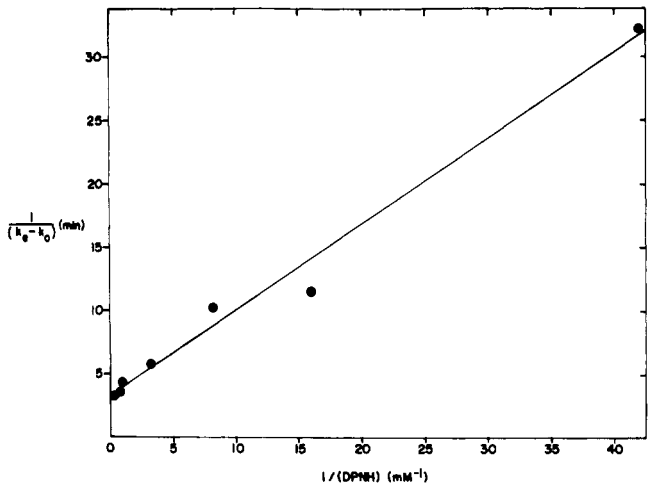


FIGURE 2: Double-reciprocal plot of the enhancement by DPNH of the rate of inactivation by 0.1 M 2,4-pentanedione. The reciprocal of the DPNH concentration is plotted vs. $1/(k_c - k_0)$, where k_c is the rate in the presence of a given DPNH concentration and k_0 is the rate without DPNH. By extrapolation of the line, a dissociation constant for the enzyme-DPNH complex (K_{DPNH}) of 0.2 mM is determined. The rate without DPNH is 3.0×10^{-2} min^{-1} and the maximal rate with saturating DPNH, 6.0×10^{-2} min^{-1}. Incubations were conducted at 30 °C as previously described, using a homogeneous enzyme preparation (specific activity > 30 units/mg) that had been purified by DEAE-cellulose and cellulose phosphate chromatography.

the values of K_{IC} and the K_m under comparable conditions suggests that the isocitrate protection results from binding in the active site area.

Effect of DPNH on Inactivation by 2,4-Pentanedione. In marked contrast to the lack of protection by DPN, DPNH accelerates the rate of 2,4-pentanedione inactivation of the enzyme. A dissociation constant for the enzyme-DPNH complex of 0.2 mM can be estimated from a plot (Figure 2) of 1/(DPNH) against the reciprocal of the incremental rate at a given DPNH level. At saturating DPNH concentrations in this set of experiments, the rate of inactivation was doubled.

In addition to enhancing the rate of 2,4-pentanedione inactivation of the enzyme, DPNH also facilitates isocitrate protection against the inactivation produced by 2,4-pentanedione (Table III). In this table, on the left-hand side, are listed rate constants for inactivation of the enzyme in the presence of a relatively high level of DPNH (1.5 mM) with and without isocitrate and/or ADP. Values for K_{IC}, calculated from eq 1 and the rate constants, are recorded in the right-hand section. In the absence of ADP, K_{IC}, which was 13 mM without DPNH, falls with increasing isocitrate from 4 to 0.7 mM at 25 mM isocitrate. The nucleotide ADP has little effect on the affinity of isocitrate for the enzyme when DPNH is present; K_{IC} reaches a limiting value of about 0.5 mM in the presence of both ADP and DPNH. In the absence of isocitrate, ADP fails to protect against the DPNH acceleration of inactivation, suggesting that these two nucleotides do not compete for identical sites.

Determination of K_I for DPNH. It seemed plausible that the apparent value of 0.2 mM for the dissociation constant for the enzyme-DPNH complex as determined from the acceleration of 2,4-pentanedione inactivation might be related to the product inhibition K_I determined from Lineweaver-Burk plots; that is, DPNH might be exerting its effect by binding to the active site. In the absence of reagent, but with all other components of the reaction mixture present, DPNH behaved

TABLE III: Protection by Isocitrate and ADP in the Presence of DPNH against Inactivation of the Enzyme by 0.1 M 2,4-Pentanedione.[a]

Isocitrate Concn (mM)	k_i (10^3 min^{-1})			K_{IC} (mM)		
	No ADP	2 mM ADP	5 mM ADP	No ADP	2 mM ADP	5 mM ADP
	50.5	45.3	56.6			
1.0	41.0	38.3		4.3	3.1	
2.5	17.5		21.7	1.3		1.6
5.0	12.1	8.31		1.6	1.1	
10.0	4.73	2.44	2.44	1.0	0.48	0.48
25.0	1.32		1.18	0.68		0.53

[a] All incubation mixtures were 1.5 mM in DPNH, 2.0 mM in $MnSO_4$, and otherwise were as described in the Materials and Methods section. The enzyme preparation was the same as that described in Figure 2.

as a linear competitive inhibitor with respect to DPN. The K_m for DPN was 86 μM, a value close to that found by Cohen and Colman (1972), and the K_I for DPNH, 32 μM. These values are also very similar to those found for bovine heart isocitrate dehydrogenase by Chen and Plaut (1963). When 0.1 M 2,4-pentanedione was present, the K_m for DPN was 55 μM, and DPNH (at concentrations up to 100 μM) functioned as a competitive inhibitor with respect to DPN, exhibiting a K_I of 60 μM. The precision of these K_I values is not sufficient to establish that K_I values of 30 and 60 μM are significantly distinct. The dissociation constant for DPNH (200 μM) determined from the enhancement of the inactivation by 2,4-pentanedione is somewhat greater than the K_I values. However, it might be expected that, just as DPNH increases the affinity between isocitrate and the enzyme, isocitrate might exert a reciprocal effect on the affinity between DPNH and the enzyme which would be reflected in a lower value of K_I as compared with the dissociation constant for DPNH.

Since DPNH is a competitive inhibitor with respect to DPN as measured in the presence of isocitrate, it is possible that DPN and DPNH would compete for binding to the enzyme in the absence of isocitrate. Were this situation to occur, it might be predicted that DPN would prevent the DPNH acceleration of the inactivation by 2,4-pentanedione. However, identical rates were obtained in the presence and absence of 2 mM DPN when the enzyme was inactivated by 0.1 M reagent in solutions containing either 0.05 or 0.5 mM DPNH. One plausible explanation might be that DPN can bind to the enzyme only when isocitrate is present. Alternatively, DPNH may bind to a separate regulatory site which is distinct from its product inhibition site, and binding at that regulatory site may be responsible for the effects of DPNH on the inactivation by 2,4-pentanedione. These possibilities cannot be distinguished by experiments of the type described here.

Attempts to Label the Enzyme with Radioactive Reagent or to Demonstrate Other Irreversible Modifications. Because of the relatively simple kinetics of inactivation and the effects of cofactor and substrate on those kinetics, it seemed reasonable that 2,4-pentanedione might be producing a specific modification of amino acid residues of the enzyme. Several attempts were made to isolate a stable derivative of the enzyme labeled with [2,4-^{14}C]-2,4-pentanedione. Although 2,4-pentanedione had been reported by Autor and Fridovich (1970)

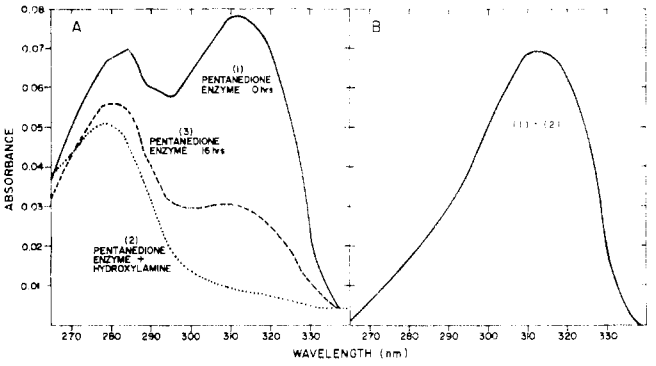


FIGURE 3: Spectra of isocitrate dehydrogenase after reaction with 2,4-pentanedione. The enzyme (0.5 mg) was incubated with 0.18 M [2,4-^{14}C]pentanedione at pH 6.15 and 15 °C until it had lost 75% of its initial activity. It was then separated from reagent by filtration at 5 °C through a Sephadex G-25 column, equilibrated with the same buffer. The fractions highest in enzymatic activity were pooled for further study. Aliquots were scanned in a Cary Model 15 spectrophotometer thermostated at 15 °C against a reference cuvette containing the same buffer. Full scale is 0.1 absorbance unit. All spectra are normalized to equal protein concentrations. (A) (Curve 1) The spectrum of modified enzyme immediately after gel filtration (solid line). (Curve 2) The enzyme from curve 1 was made 0.1 M in hydroxylamine. The spectrum shown here was attained after 10 min (dotted line). (Curve 3) This spectrum (dashed line) resulted from incubation of a sample of enzyme at 15 °C for 16 h. (B) The difference spectrum generated by subtraction of curve 2 from curve 1. The enzyme described in Figure 2 was used in these experiments.

to form an enamine with the lysyl residues of acetoacetate decarboxylase, other reactions seemed possible, such as the formation of a Schiff base with lysyl groups or of an adduct with arginine (Gilbert and O'Leary, 1975). In order to avoid complications from side reactions which might arise secondarily as a result of protein denaturation, all reactions were terminated when the residual activity reached 25% that of the original enzyme or at an equivalent reaction time in the case of enzyme incubated in the presence of protecting ligands. Three possibilities were explored: the trapping of a stable product by rapid filtration on a column of Sephadex G-25 in 50 mM ammonium bicarbonate buffer, reduction of a postulated Schiff base by treatment with an excess of sodium borohydride, followed by dialysis, and, by analogy to the stabilization of the arginine-2,3-butanedione adduct by acid (Riordan, 1973), addition of an equal volume of 2 N HCl to the reaction mixture with subsequent dialysis against 1 N HCl. The reagent used in all cases was [2,4-^{14}C]-2,4-pentanedione. After the first procedure, the only one in which the enzyme was rapidly separated from excess reagent, the enzyme seemed to have retained an amount of radioactivity equivalent to 1 mol per subunit; however, the label was lost during the repeated evaporation of the buffer. No significant radioactivity could be detected at the conclusion of the other procedures. A Schiff base was thus clearly ruled out, as was the type of adduct formed with 2,3-butanedione. These experiments did suggest the formation of a slowly dissociable complex such as an enamine.

A possibility remained that 2,4-pentanedione might modify some enzyme residues without itself remaining bound. Amino acid analyses of 75% inactivated enzyme, enzyme reacted for the same length of time (about 150 min at 15 °C) in the presence of 2 mM $MnSO_4$ and 50 mM isocitrate, and enzyme control without 2,4-pentanedione showed no significant differences from previously published values (Shen et al., 1974). Therefore, under these reaction conditions no residue appears to be converted to an acid-stable derivative by 2,4-pentanedione treatment. However, when a sample of enzyme was allowed to react for 24 h with reagent and was then subjected to amino acid analysis, the arginine content was reduced, and an asymmetry was apparent in the lysine peak. This observation is consistent with the report of Gilbert and O'Leary (1975) that 2,4-pentanedione reacts slowly with arginine to form a pyrimidine derivative that is decomposed to ornithine by acid hydrolysis. Ornithine coelutes with lysine upon amino acid analysis. Thus 2,4-pentanedione does react with arginyl residues in isocitrate dehydrogenase, but at a rate that is too slow to account for the observed inactivation.

Formation of Modified Enzyme with a New Absorption Spectrum. Tagaki et al. (1968) reported that reaction of acetopyruvate with the lysyl residues of acetoacetate decarboxylase led to an enamine δ which exhibited an ultraviolet absorption peak at 315 nm. In order to investigate the possible formation of an enamine in isocitrate dehydrogenase, scans between 320 and 350 nm were repeatedly carried out on a reaction mixture. The absorbance increased concomitant with inactivation of the enzyme. After the reaction mixture had been filtered through Sephadex G-25 to remove reagent, a new absorbance peak with a maximum at 312 nm was apparent. Thus, the reaction product was sufficiently stable to be isolated. The stoichiometry of the modification reaction and the extinction coefficient of the product were both determined by use of radioactive 2,4-pentanedione. The absorption spectrum of the modified enzyme is shown in curve 1 of Figure 3A. When this enzyme was made 0.1 M in hydroxylamine, which Gilbert and O'Leary (1975) reported to dissociate the enamine of 2,4-pentanedione with the lysines of lysozyme, the band at 312 nm disappeared rapidly to yield the spectrum of curve 2, Figure 3A. The concentration of 2,4-pentanedione in the enzyme solution was determined from its radioactivity and the concentration of protein from the absorbance of curve 2 at 280 nm, yielding a value of 2.2 mol of reagent incorporated per peptide chain for 75% inactivated enzyme. If it is assumed that the extent of incorporation is linearly related to the degree of inactivation, reaction of an average of 2.9 residues would be required for complete inactivation. Two molar extinction coefficients, 2.0×10^4 M^{-1} cm^{-1} at 312 nm and 1.4×10^4 M^{-1} cm^{-1} at 325 nm, were calculated from the absorption spectrum in curve 1 and the concentration of 2,4-pentanedione. An extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} at 325 nm can be estimated from the data reported by Tagaki et al. (1968) for studies of model enamines, and the similarity of the two values is supportive of the conclusion that curve 1 (Figure 3A) indeed represents an enamine spectrum. Moreover, subtraction of curve 2 from curve 1 leads to Figure 3B which is strikingly similar to the spectrum of the enamine (Gilbert and O'Leary, 1975) of 2,4-pentanedione and *n*-butylamine. The spectrum in Figure 3B is different from that of the adduct of 2,4-pentanedione with arginyl residues, which has a maximum at 305 nm (Gilbert and O'Leary, 1975).

Stability of the Enamine of Isocitrate Dehydrogenase. An aliquot of the reacted and gel-filtered enzyme pool from the previous section was monitored at 312 nm for several hours at 14 °C. The rate of decrease of A_{312nm} was logarithmic and exhibited an apparent first-order rate constant (assuming an end point of zero) of 1.65×10^{-3} min^{-1}. The spectrum of this enzyme sample after 16 h at 14 °C is shown in curve 3 of Figure 3A. Addition of sodium borohydride had no effect on the remaining absorbance.

The abolition of the 312-nm absorbance band (Figure 3A, curve 1) by hydroxylamine, which was complete within 10 min at 15 °C, seemed to be biphasic. At least 60% of the 312-nm

absorbance was lost with a first-order rate constant of 2 min^{-1}, and the balance with a rate constant of 0.2 min^{-1}. Restoration of enzymatic activity was much slower than the loss of 312-nm absorbance. The maximum recovery of activity was achieved when G-25-filtered enzyme (after 75% inactivation) was made 0.1 M in hydroxylamine, and incubated at 15 °C. (The measured enzyme activity was corrected for the decay of the same sample, untreated with hydroxylamine, and incubated at the same time.) The activity tripled in 2 h, and 64% of the predicted recovery was achieved. Since unreacted enzyme loses activity when incubated with hydroxylamine, it is unlikely that total reactivation could be attained. The fact that abolition of enamine absorbance is associated with regain of activity strongly suggests that the enamine formation was responsible for the inactivation produced by 2,4-pentanedione.

Enamine Formation in the Absence and Presence of Isocitrate. The increase in absorbance of an incubation solution was monitored at 325 nm until an end point of 0.105 absorbance unit was reached. This end point is equivalent to the formation of 3 enamine groups per protein chain, a value in good agreement with that estimated previously. First-order plots of the absorbance change and of activity loss are shown in Figure 4. The rate constants at 15 °C for inactivation (line A) and for the change in A_{325nm} (line B) were 7.1×10^{-3} and 5.9×10^{-3} min^{-1}, respectively. The data of Figure 4 are suggestive of the association of enamine formation with inactivation.

Although isocitrate protects against inactivation by 2,4-pentanedione, it does not abolish enamine formation. Incubation of the enzyme with 2,4-pentanedione in the presence of 50 mM DL-isocitrate and 2 mM $MnSO_4$ leads to the formation of 2 mol of enamine per subunit at a first-order rate that is five to ten times as rapid as the rate of inactivation. For example, the 325-nm absorbance of a solution of isocitrate dehydrogenase containing 50 mM DL-isocitrate and 0.1 M 2,4-pentanedione was monitored at 15 °C until it appeared to stabilize after 6 h. The total absorbance change corresponded to an enamine formation of 2 mol per subunit, and 40% of the enzymatic activity was lost. When this reaction mixture was filtered through Sephadex G-25, the number of enamine groups per peptide chain was confirmed from its absorbance at 312 nm as 2. The rate constants were 7.1×10^{-3} and 1.5×10^{-3} min^{-1} for the A_{325nm} change and the activity loss, respectively. It is thus plausible that an average of one residue is protected by isocitrate from reaction with 2,4-pentanedione. If this average of one represents a unique residue, rather than partial reaction at several groups, this may be the only one essential to activity.

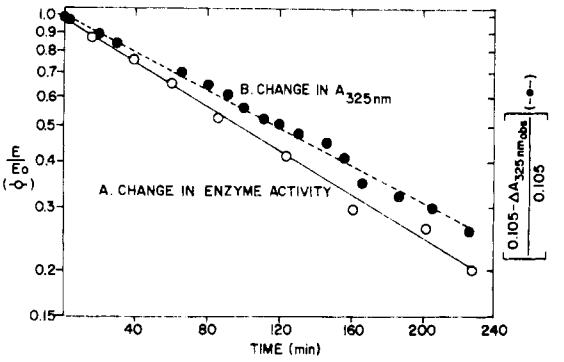


FIGURE 4: The inactivation of DPN-dependent isocitrate dehydrogenase (0.1 mg/mL) at 15 °C by 0.1 M 2,4-pentanedione and the concomitant increase in absorbance at 325 nm. The incubation was carried out in a 1.0-mL cuvette, as described in Materials and Methods, and samples were removed at intervals for determination of enzymatic activity, which is plotted (line A) as the ratio of the active residual enzyme at a given time to the active enzyme at zero time (E/E_0). The absorbance at 325 nm, which was read from a recorder tracing, approached an end point of 0.105 and is plotted (line B) as the fraction $(0.105 - \Delta A_{325nm})/0.105$ vs. time. The enzyme described in Figure 2 was used in these experiments.

Discussion

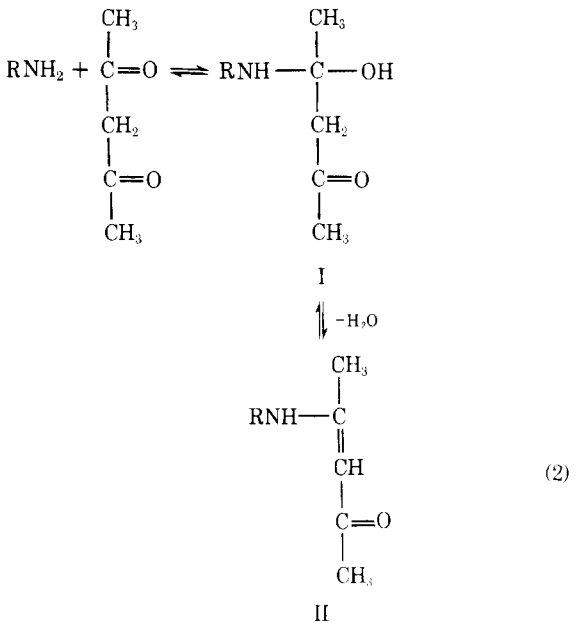
The experiments presented in this paper indicate that the pig heart DPN-dependent isocitrate dehydrogenase is specifically inactivated by covalent reaction of 2,4-pentanedione with one to three lysyl residues of the enzyme. Modification does not occur within the binding site for either the coenzyme or for the allosteric activator since neither DPN nor ADP protects against the inactivation. Indeed the reduced coenzyme, DPNH, enhances the rate of inactivation by 2,4-pentanedione, implying that the binding of DPNH by the enzyme leads to the increased availability or reactivity toward 2,4-pentanedione of the essential lysyl residues. In contrast, significant protection against inactivation is provided by isocitrate when present together with manganous ion, suggesting that modification takes place at a binding site for the substrate and metal ion. Although isocitrate in the absence of Mn^{2+} can bind to the enzyme, as evidenced by its ability to retard spontaneous denaturation of the enzyme, free isocitrate does not yield appreciable protection against the inactivation produced by 2,4-pentanedione. Similarly, manganous ion alone produces only a threefold decrease in the rate of inactivation, supporting the concept that both metal ion and isocitrate must combine with enzyme to produce a functional complex. Furthermore, ADP reduces to about the same extent the values of the K_m and K_{IC} for isocitrate. All of these observations are consistent with reaction of 2,4-pentanedione within a specific manganous-isocitrate binding site. (As is the case in most chemical modification studies, one cannot exclude the possibility that binding of substrate induces a conformational change which results indirectly in a reduced rate of modification at a distant site on the enzyme.)

The concentrations of DL-isocitrate have been expressed in this paper in terms of total isocitrate even though it has been shown that dibasic isocitrate is the actual substrate for DPN-dependent pig heart isocitrate dehydrogenase (Cohen and Colman, 1974). The concentration of free manganous ion has been shown to control whether free isocitrate predominantly binds to a Mn-enzyme complex or whether the Mn-isocitrate complex predominantly combines with enzyme. However, even though 2,4-pentanedione is an effective chelator of manganous ion, the presence of reagent increases the K_m for isocitrate by only 50%. It is therefore likely that the same form of isocitrate binds with and without reagent.

The effects of nucleotides on the inactivation of DPN-dependent isocitrate dehydrogenase by 2,4-pentanedione may help to elucidate how these compounds bind to the enzyme. ADP has previously been shown (Chen et al., 1964) to cause aggregation of bovine heart DPN-dependent isocitrate dehydrogenase whereas DPNH prevented the ADP-induced aggregation. In the present study both ADP and DPNH have been found to enhance the effectiveness of isocitrate protection against inactivation. At saturating DPNH levels, ADP does not appear to cause a significant further reduction in K_{IC}. Moreover, the effect of DPNH is unique in its acceleration of the rate of 2,4-pentanedione inactivation. Despite the fact that both ADP and DPNH exert similar effects on isocitrate binding, they appear to occupy separate binding sites because ADP cannot prevent the DPNH rate enhancement. These findings are similar to those of Plaut and Aogaichi (1968) and

Harvey et al. (1972). In the earlier paper, studies on DPN-dependent isocitrate dehydrogenase from pig liver revealed that ADP failed to prevent inhibition by DPNH. In the more recent paper (Harvey et al., 1972) an exceptionally tight single binding site for DPNH was discovered in the bovine heart enzyme; ADP had no effect on this binding. The dissociation constant of 200 μM for DPNH that was determined in the present investigation from the acceleration of 2,4-pentanedione inactivation is 100 times weaker than the value for the single site described by Harvey et al., and thus it is unlikely that the two constants relate to analogous sites. However, the pattern of separate sites for DPNH and ADP appears consistent. Whether DPNH exerts its inactivation rate enhancing effect by binding to its product site or to a separate regulatory site cannot at present be determined.

Inactivation by 2,4-pentanedione is clearly the result of the formation of enamines of a relatively few lysyl residues: no more than an average of 3 out of a total of 26 per subunit. The isolation of an enamine-containing inactive enzyme and the reactivation with hydroxylamine are consistent with the requirement of lysyl groups for activity, although it is also possible that terminal amino groups may react with the reagent. The small discrepancy between the rate of inactivation and the slower rate of absorbance change may indicate that inactivation is caused by the initial reaction of the ϵ-amino group of lysine and 2,4-pentanedione to form a carbinolamine I, which is dehydrated slightly more slowly to yield an enamine II in accordance with the following mechanism:



(2)

Alternatively, the absorbance rate data may not be sufficiently accurate to differentiate between reaction with the residue essential to activity and with the other lysyl residues. The lag between abolition of enamine absorbance and restoration of enzymatic activity after addition of hydroxylamine implies that a conformational change in the enzyme occurs subsequent to liberation of lysyl residues.

The reaction of 2,4-pentanedione with lysyl residues in DPN-dependent isocitrate dehydrogenase is both faster and more selective than are the reactions reported by Gilbert and O'Leary (1975) with *n*-butylamine and with the lysines of lysozyme. The second-order rate constant for inactivation at 30 °C of isocitrate dehydrogenase, 0.28 M^{-1} min^{-1}, is 140 times that reported for the reaction at 21 °C of 2,4-pentanedione with *n*-butylamine (Gilbert and O'Leary, 1975). In striking contrast to the slow rates reported for the reaction of 2,4-pentanedione with the lysines of lysozyme are the rates of reaction of β-diketones with acetoacetate decarboxylase (second-order rate constants at 0 °C from 2×10^4 M^{-1} min^{-1} for reaction with the substrate analogue, acetopyruvate, to 4×10^2 M^{-1} min^{-1} for interaction with 2,4-pentanedione) (Tagaki et al., 1968; Autor and Fridovich, 1970). The limited reaction of 2,4-pentanedione with a few lysines of isocitrate dehydrogenase falls closer in rate to the slow reaction of the lysines of lysozyme than to the extremely rapid rate of the single lysine in acetoacetate decarboxylase. Nevertheless, the selectivity of the reaction in isocitrate dehydrogenase and the relative rapidity with which it occurs imply that these lysyl residues differ from normal lysines, either in pK or in accessibility to the reagent.

A lysine residue has previously (Shen and Colman, 1975) been implicated in the active site region of the pig heart DPN-dependent isocitrate dehydrogenase as a result of studies of modification of the enzyme by cyanate. In that case it was postulated that the active species in the carbamoylation reaction was isocyanic acid, which acts as an analogue of carbon dioxide, the product of the oxidative decarboxylation of isocitrate. The similarity in size between 2,4-pentanedione and isocitrate suggests the possibility that this reagent might also act as a substrate analogue. However, it does not appear that a dissociable complex is formed between reagent and enzyme prior to inactivation because the rate of inactivation is linearly dependent on reagent concentration. Nevertheless this finding does not rule out a specific reaction of the reagent at the substrate binding site. Because of the reversibility of the 2,4-pentanedione reaction, it is not at present feasible to determine whether cyanate and 2,4-pentanedione react with the same or distinct lysine residues. Functions for more than one lysine residue in the enzymatic reaction may be postulated: it is possible that one lysine facilitates the binding of substrate by electrostatic interaction with the carboxylate groups of isocitrate, while another lysine participates directly in catalysis as a general acid or general base (Shen and Colman, 1975). Fan and Plaut (1974) attributed the inactivation of bovine heart DPN-specific isocitrate dehydrogenase by several aldehydes to modification of a single amino group per enzyme subunit. This amino group may be analogous to the lysine residues of the pig heart enzyme which react with cyanate and/or 2,4-pentanedione.

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Magnetic Resonance Studies on Manganese–Nucleotide Complexes of Phosphoglycerate Kinase†

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ABSTRACT: Measurements of the relaxation rate of water protons (PRR) have been used to study the interaction of yeast phosphoglycerate kinase with the manganous complexes of a number of nucleotides. The results indicate that phosphoglycerate kinase belongs to the same class of enzymes as creatine kinase, adenylate kinase, formyltetrahydrofolate synthetase, and arginine kinase, with maximal binding of metal ion to the enzyme in the presence of the nucleotide substrate. However, an analysis of titration curves for a number of nucleoside diphosphates (ADP, IDP, GDP) showed that there is a substantial synergism in binding of the metal ion and nucleotide to the enzyme in the ternary complex. The metal–substrate binds to the enzyme approximately two orders of magnitude more tightly than the free nucleotide. Other evidence for an atypical binding scheme for Mn(II)–nucleoside diphosphates was obtained by electron paramagnetic resonance (EPR) studies; the EPR spectrum for the bound Mn(II) in the enzyme–MnADP complex differed substantially from those obtained for other kinases. An identical EPR spectrum is observed with the MnADP complex with the rabbit muscle enzyme as with the yeast enzyme. In contrast, the dissociation constant for the enzyme–MnATP complex is approximately fourfold lower than that for enzyme–ATP, and there are no substantial changes in the electron paramagnetic resonance spectrum of $MnATP^{2-}$ when the complex is bound to phosphoglycerate kinase. A small but significant change in the PRR of water is observed on addition of 3-phosphoglycerate (but not 2-phosphoglycerate) to the MnADP-enzyme complex. However, addition of 3-phosphoglycerate to enzyme–MnADP did not influence the EPR spectrum of the enzyme-bound Mn(II).

Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) catalyzes the reaction, $MATP^{2-}$ + 3-phosphoglycerate ⇌ $MADP^{-}$ + 1,3-diphosphoglycerate, where the divalent metal ion may be Mg^{2+}, Mn^{2+}, Ca^{2+}, or Co^{2+}. The enzyme has been isolated from a number of sources (see Scopes, 1971, for a review), though it is the enzyme from yeast that has been subjected to the most detailed investigation. For example, much work has been done on the kinetics of the yeast enzyme (Larsson-Razniekiewicz, 1964, 1967, 1970; Krietsch and Bücher, 1970). Recently, extensive x-ray crystallographic studies have been carried out (Wendell et al., 1972; Bryant et al., 1974) and high resolution NMR[1] studies of the enzyme–substrate complexes have been reported (Tanswell et al., 1976).

In this paper, we present the results of magnetic resonance studies on complexes formed between the enzyme and various Mn–nucleotide complexes. Results from PRR measurements of water indicate an unusual synergism in the binding of Mn–nucleoside diphosphates but not Mn–nucleoside triphosphates to the enzyme. These differences are reflected in qualitative differences in the respective EPR spectra. Evidence from both techniques for the formation of an abortive quaternary complex, enzyme–MnADP–3-phosphoglycerate, is also presented. A preliminary account of some of this work has appeared elsewhere (Chapman et al., 1974).

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[1] Abbreviations used: NMR, nuclear magnetic resonance; PRR, proton relaxation rate; EPR, electron paramagnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; T_{1p}, the paramagnetic contribution to the longitudinal proton relaxation time of water; T_{1M}, the longitudinal proton relaxation time of water in the first coordination sphere of Mn(II); ϵ_a, ϵ_b, and ϵ_t are the characteristic PRR enhancement factors for the Mn(II)–substrate, Mn(II)–enzyme, and Mn(II)–substrate–enzyme complexes, respectively; 3-PGA, 3-phosphoglycerate; ω, the nuclear Larmor precessional frequency; τ_c, correlation time for a Mn(II) complex; τ_r, the rotational correlation time; τ_e, the electron spin–lattice relaxation time; τ_M, the residence time of a water molecule in the first coordination sphere of Mn(II); XDP, xanthosine diphosphate; Ap(CH_2)p, α,β-methylene analogue of ADP; App(CH_2)p, β,γ-methylene analogue of ATP.