Scheme I

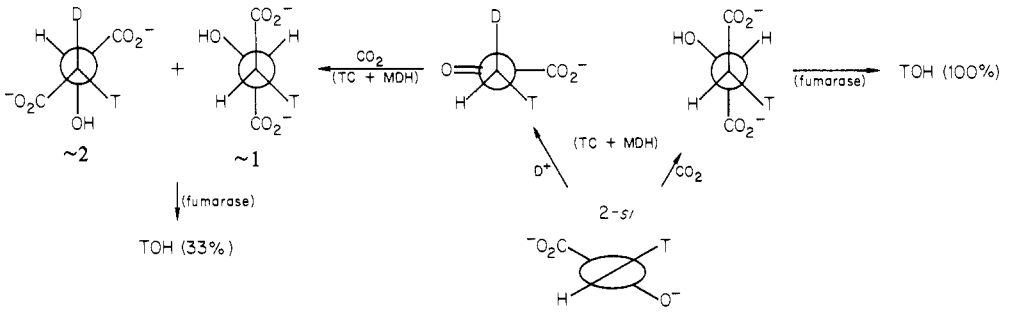

Table II. Stereochemistry of Transcarboxylase Reaction with Enolpyruvate

reaction conditions[a] (form of PEP-3-T)	tritium removed by fumarase (%)	
	no LDH	+LDH[b]
Z	37.1 ± 0.8 (6)	36.0 ± 0.3 (6)
Z[c]	49.0 ± 0.5 (7)	50.6 ± 0.2 (6)
E	64.3 ± 0.5 (6)	63.6 ± 0.3 (4)
E = *Z*	49.7 ± 0.3 (3)	50.6 ± 0.2 (6)

[a] Each incubation was contained in 0.5 mL of D_2O (at pD 6.0, 20 °C): Na maleate (40 mM), NADH (2 mM), malate dehydrogenase (20 units), transcarboxylase (0.23 units), PEP-3-T (*Z*, *E*, or *E* = *Z*, as noted, 0.8 mM), (*RS*)-methylmalonyl-CoA (4 mM), acid phosphatase (0.016 units) and lactate dehydrogenase, LDH (13 units or none as noted). The reactions were monitored at 340 nm and were followed to completion. (*E*)- and (*Z*)-PEP-3-T were prepared as before,[10] and malate was isolated and analyzed for purity and position of tritium as before.[6a] In all cases the malate was radiochemically >95% pure as shown by its ability to give up its tritium to water upon prolonged incubation with malate dehydrogenase.[6a] The number of determinations of counts made volatile by fumarase is shown in parentheses. [b] With LDH only 5-10% of the reaction yielded oxalacetate (or malate). [c] Avidin present at 20 μM.

Because protonation is much more rapid than carboxylation, the results shown in Table II when lactate dehydrogenase was omitted are those expected for chiral pyruvate. In fact, they agree with values obtained for pyruvate that was formed by action of pyruvate kinase and ADP on PEP-3-T in D_2O.[6a,9] D^+ addition is made to the 2-*si* face of enolpyruvate: (3*S*)-pyruvate is formed from (*Z*)-PEP-3T and (3*R*)-pyruvate from (*E*)-PEP-3-T. With avidin present no distinction was made between the two faces of enolpyruvate, as is consistent with the loss of enzyme function. In the presence of lactate dehydrogenase (13 units compared with ~0.23 units of transcarboxylase) all free pyruvate would be trapped immediately as lactate, and any malate formed would have come from direct carboxylation of enolpyruvate *and* carboxylation of enolpyruvate derived from pyruvate that had not yet dissociated from the enzyme. The similarity of results in which malate dehydrogenase was present with and without lactate dehydrogenase seems to show that *direct* carboxylation of enolpyruvate is negligible. Were it to occur it would raise the counts exchanged by fumarase when (*Z*)-PEP-3-T was used and lower them in the case of (*E*)-PEP-3-T relative to the values obtained without lactate dehydrogenase.

Observations almost identical with these have been made by using rat liver pyruvate carboxylase. In that case stereospecific ketonization of enolpyruvate occurred only in the presence of the reagents necessary to carboxylate the biotin: ATP, HCO_3^-, and acetyl-CoA. As with the transcarboxylase, direct carboxylation of enolpyruvate could not be observed by stereochemical means.

Although the evidence given here in support of a carbanion mechanism would undoubtedly be more complete if direct carboxylation of enolpyruvate had been demonstrated, simple kinetic parameters of the reaction might prevent this step from being observed by a stereochemical approach. Slow release of oxalacetate relative to ketonization and liberation of pyruvate would readily explain the observations. It would be surprising to discover that the avidin-sensitive ketonization was a side reaction unrelated to the carboxylation of pyruvate: ketonization requires that the enzyme be carboxylated, is stereospecific, and forms pyruvate at a site from which its carboxylation can be observed without prior dissociation from the enzyme.

It may be of interest to note that in all reactions of PEP that may be expected to use enolpyruvate as an intermediate, addition to C-3 occurs from the 2-*si* face.[10,11] The pyruvate-activating subunits of transcarboxylase and pyruvate carboxylase acting on enolpyruvate directly are now known to continue this generalization.

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Registry No. E.C.2.1.3.1, 9029-86-1; enolpyruvate, 19071-34-2; methylmalonyl-CoA, 1264-45-5.

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Evidence for Two Types of Binding Sites in Cadmium Metallothionein Determined by Perturbed Angular Correlation of γ Rays[1]

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Metallothionein is a widely distributed metal- and sulfur-rich low molecular weight (~6800) protein which is of considerable interest in view of its presumed involvement in metal metabolism, homeostasis, and detoxification.[2] All mammalian forms (including man) characterized to date contain a single polypeptide chain of a total of 61 amino acids, out of which 20 are cysteine residues and serve as binding ligands for all metal ions.[3] Recent ^{113}Cd

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Table I. Nuclear Quadrupol Interaction (NQI) Parameters for Different ^{111}Cd(II)-Metallothionein Derivatives

^{111}Cd(II)-metallothionein derivatives[a]	ω_1, MHz	η_1	ω_2, MHz	η_2	P_1, %	P_2, %	δ_1, %[b]
uniformly labeled ^{111}Cd(II)-metallothionein	116 ± 6	0.55 ± 0.07	579 ± 32	0.51 ± 0.09	84 ± 4	16 ± 4	20
carrier-free ^{111}Cd added to apometallothionein[c]	119 ± 4	0.61 ± 0.05	558 ± 23	0.55 ± 0.07	81 ± 6	19 ± 6	20
1 equiv of ^{111}Cd added to Zn,Cd-metallothionein	149 ± 5	0.30 ± 0.03	714 ± 22	0.74 ± 0.04	81 ± 4	19 ± 4	25

[a] See text for details. [b] The frequency ω_1 is broadened by $e^{-1/2\delta_1^2\omega_1^2t^2}$ (see ref 9). [c] 10^{-12} mol of carrier-free ^{111}Cd were added to 5 mL of 10^{-4} M apometallothionein.

NMR[4] as well as ESR and magnetic susceptibility measurements on Co(II)-metallothionein[5] strongly suggest the existence of a unique metal–thiolate cluster structure in this protein. However, additional knowledge on the details of molecular architecture of the seven metal-binding sites in metallothionein (~6–7 g-atoms/mol, usually Zn^{2+} and Cd^{2+}) is required in order to establish a realistic model of the molecule and to understand its function.

Since metallothionein is the only known protein in which cadmium is found naturally,[6] perturbed angular correlation of γ rays (PAC) employing ^{111}Cd nuclei offers itself particularly for such a study. Analysis of PAC spectra using ^{111}Cd has proven to be useful in the structural elucidation of specific metal sites in metalloproteins under both crystalline[7] and noncrystalline[8] conditions. We report here PAC measurements performed on fully and partially saturated ^{111}Cd metallothionein.

In PAC experiments,[9] an excited metal nucleus functions as a spectroscopic probe. Excited ^{111}Cd is decaying to the ground state via an intermediate state of $T_{1/2}$ = 84 ns. During this process two γ rays are emitted. They are related to each other by an angular correlation function $W(\theta,t)$, where θ is the angle between γ_1 and γ_2 and t is the delay time of γ_2 with respect to γ_1. If this intermediate level interacts with its surroundings, i.e., is split by hyperfine interaction, then the angular correlation function of nonmagnetic Cd ions is perturbed by the nuclear quadrupole interaction (NQI). The result of a PAC measurement is then a time spectrum showing the development in time of the hyperfine interaction frequencies.[9]

PAC spectra were recorded with a four-detector slow–fast coincidence spectrometer. The coincident counting ratio $W(\theta,t)$ was measured at fixed angles 0–180 and 0–90°, as a function of the delay time t. From coincident rates which were determined experimentally, $W(180°,t)$ and $W(90°,t)$, the NQI parameters ω and η were evaluated according to the equation[7]

$$\frac{W(180°,t)}{W(90°,t)} = \frac{1 + \sum_i A_i G(t,\omega_i,\eta_i)}{1 - \frac{1}{2}\sum_i A_i G(t,\omega_i,\eta_i)} \quad (1)$$

where A_i is the partial amplitude for a NQI parameter set (ω_i,η_i) and $G(t,\omega_i,\eta_i)$ is a corresponding perturbation factor.[8,9] The NQI parameters were derived from least-squares fits to the $W(180°,t)/W(90°,t)$ ratio. Rabbit liver metallothionein-1 was isolated and characterized by the established method.[10] Thionein (apoprotein) was prepared by dialyzing Zn/Cd–metallothionein (45% Zn, 55% Cd) against three changes of 0.1N HCl. Protein concentration was determined spectrophotometrically in 0.1N HCl, with an absorption coefficient A_{220} = 7.9 cm^{-1} mL^{-1}.[11] The ^{111}Cd isotope was produced by bombardment of metallic Pd (98% enriched ^{108}Pd) with 21-MeV α particles. The ^{111}Cd isotope exists in its 497-keV excited state with $T_{1/2}$ of 49 min. ^{111}Cd was separated (about 10^{-12} mol) from ^{108}Pd as described elsewhere.[8] Subsequently, this amount of ^{111}Cd was mixed with cold Cd^{2+} carrier and added in the desired proportion of 10^{-4} M thionein (apoprotein) at pH 1. Subsequently, the solution was degassed and, under argon atmosphere, its pH adjusted to the desired value with Trizma base. In samples containing the full complement of 7 equiv of Cd^{2+}/mol, the pH was brought to 8.0.

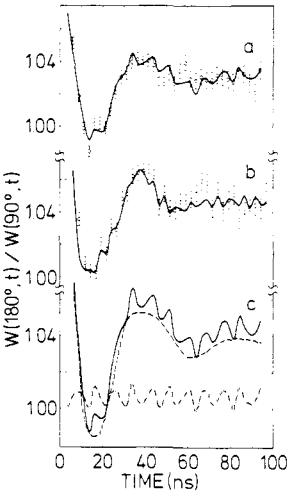

Figure 1. PAC time spectra ($W(180°)/W(90°)$) for ^{111}Cd in various derivatives of metallothionein in 65% (weight) sucrose, 0 °C. The fully drawn curves represent least-squares fits to the spectra. The bars indicate ±1 standard deviation. In all cases the viscosity of the sucrose solution has immobilized the protein within the time scale of the experiment: (a) fully reconstituted ^{111}Cd(II)–metallothionein, pH 8.0; (b) 1 equiv of ^{111}Cd added to Zn,Cd–metallothionein, pH 8.0 (for details see Table I); (c) resolution of the least-squares fit of the spectrum in a (—) yields two NQI's with parameters ω_1 = 116 MHz, η_1 = 0.55 (---), and ω_2 = 579 MHz, η_2 = 0.51 (-·-) (see Table I). Because of the finite time resolution of the PAC spectrometer (τ = 3.3 ns), the high frequency NQI, ω_2, (see Table I) is strongly damped. Hence, because of growing statistical noise as a function of time, the least-squares fits to all spectra were restricted to a time window between 0 and 40 ns. However, all fitted PAC spectra were examined for consistency with the derived NQI parameters up to 100 ns.

In samples where carrier-free ^{111}Cd was added to thionein (10^{-10} M ^{111}Cd/10^{-4} M thionein), the pH was brought only to pH 5.0 to avoid possible oxidation of the bulk of unoccupied thiolate ligands during the measurement. Under these conditions Cd^{2+} is bound quantitatively to the apoprotein.[12] In some experiments, 1 equiv of Cd carrier containing ^{111}Cd was added directly to native (Zn,Cd)-metallothionein at pH 8 causing replacement of less firmly bound zinc ions.[12] The displaced zinc ions were subsequently removed by treatment with Chelex 100. In all experiments the concentration of the excited ^{111}Cd isotope in the sample was of the order of 10^{-10} M. Unless stated otherwise, the designation ^{111}Cd refers to the carrier-containing material. All measurements were performed in 65% sucrose at 0 °C. So that traces of metal contaminants could be removed, all solutions were passed over a column of Chelex 100. All experiments were carried out in duplicate or in triplicate.

The PAC measurement of uniformly ^{111}Cd labeled metallothionein (Figure 1a) revealed two characteristic frequencies, ω_1 at about 120 MHz and ω_2 at about 580 MHz, with amplitude

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equal to 80% and 20%, respectively (see Table I). The occurrence of two vastly different frequencies, ω_1 and ω_2, is consistent with the existence of two different coordination geometries for Cd ions in metallothionein. Since pure T_d or O_h symmetries produce a spectrum lacking NQI, our results must indicate distortion from either symmetry. Of these two options, recent spectroscopic studies[12,13] greatly favor a T_d-derived symmetry.[10,14] Interestingly, the same two frequencies with the same relative amplitudes were also seen in an experiment where 10^{-12} mol of ^{111}Cd were added to a vast excess of the metal-free protein (10^{-4} M) (Figure 1b, Table I). Since under these conditions cluster structures do not form, one can conclude that these two different types of binding sites are not affected by changes in the outer coordination sphere of the complex and, hence, by the occupation of the neighboring metal-binding sites. That the participation of the same mercaptide group to one or several Cd ions does not have to affect the first coordination sphere geometry is in keeping with the constant Cd-S binding distance of about 2.5-2.55 Å irrespective of whether S is coordinated to one or two Cd ions.[15-17] The 120-MHz frequency ω_1 corresponds in order of magnitude to the 65-MHz frequency displayed by ^{111}Cd substituted for zinc in the crystallographically defined structural metal site of liver alcohol dehydrogenase. In this site, four cysteinyl thiolate ligands are arranged in a coordination geometry very close to that of a regular tetrahedron.[18] Its presence in metallothionein thus indicates that 80% of the binding sites have a similar, only weakly distorted T_d symmetry. By contrast, the 580-MHz frequency must arise from site(s) with entirely different geometric properties. A square-planar CdS_4^{2-} complex would exhibit a frequency of about 880 MHz.[18] Additional filling up to an octahedron, e.g., with either water or carboxyl ligands, would result in frequencies of about 400 and 640 MHz, respectively. Thus, the 580-MHz frequency could originate either from an extremely distorted tetrahedral geometry approaching square-planar coordination or, less likely (vide supra), from a distorted octahedral geometry with four planar mercaptide ligands and, for example, two carboxyl ligands in the remaining axial positions. Similarly, no feature characteristic of penta- or hexacoordination has been observed thus far in the spectroscopic studies of Co(II)-metallothionein.[5,10,14]

All spectra indicate about a 20% frequency broadening[9] associated with the 120-MHz frequency. Such an effort can arise either from a distribution in position of several charged protein groups within 5 Å of the cadmium atom or from the existence of minor differences in the local metal geometries. The latter would indicate an amplitude enhancement ("beat") for seven or less NQI's at larger t. Its lack suggests that the former is the case of the observed broadening. It is tempting to view the proximity of the lysine residues of metallothionein to the metal-complexing Cys-X-Cys sequences as a cause for such charge fluctuation.[20] The positive charges of the lysine residues have been postulated to serve as a role in compensating the negative charges associated with each thiolate site.[3,21]

In contrast to the 120-MHz signal, the 580-MHz signal is sharp. Its relatively small amplitude P_2 (see Table I) may indicate that it arises from about one cadmium binding site.

A replacement of 1 equiv of Zn in (Zn,Cd)-metallothionein by ^{111}Cd also results in the appearance of two NQI. Since under such circumstances ^{111}Cd replaces a zinc ion,[12] one can conclude, albeit indirectly, that zinc-like cadmium ions can occupy both types of binding sites in the protein. However, these NQI are shifted significantly toward higher frequency values, i.e., ω_1 = 149 MHz and ω_2 = 714 MHz (see Table I). This is best explained by additional geometrical distortion imposed upon the overall protein structure due to the differences in the sulfur-metal distances in the crystallographically defined cadmium and zinc model complexes. It is interesting that differences between solely Cd-containing forms of metallothionein and mixed (Cd- and Zn-containing) forms are also noted in their respective CD spectra. The Cd-induced ellipticity bands are much larger in the mixed form, suggesting a distorting influence on the coordination of Cd-thiolate complexes in the mixed-metal environment within the metal-thiolate cluster.[11,19,22]

In summary, the PAC data have shown that the majority of the metal-binding sites exhibit weakly distorted tetrahedral geometry, but that these are also site(s) with an entirely different metal geometry.

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Reactions of Excited Triplet Diphenylcarbene Studied with Picosecond Lasers

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The reactivities of the lowest singlet and triplet states of carbenes with alcohols and olefins in fluid solution have been extensively investigated.[1-11,17] For the specific case of diphenylcarbene (DPC) it has been shown that it reacts preferentially from its lowest singlet state with methanol, apparently by direct insertion into the OH bond of the alcohol.[1,2,4] The triplet ground state of DPC, on the other hand, has been shown to react with far greater ease toward

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