Kirby, A. J. (1983) *The Anomeric Effect and Related Stereoelectronic Effects at Oxygen*, pp 1-149, Springer-Verlag, Berlin.
Kossiakoff, A. A., & Spencer, S. A. (1981) *Biochemistry 20*, 6462.
Kossiakoff, A. A. Chambers, J. L., Kay, L. M., & Stroud, R. M. (1977) *Biochemistry 16*, 654.
Lee, W., & Turnbull, J. H. (1961) *Experientia 17*, 360.
Lehn, J. M., & Wipff, G. (1976) *J. Am. Chem. Soc. 98*, 7498.
Lerner, D. B., & Kearns, D. R. (1980) *J. Am. Chem. Soc. 102*, 7612-7613.
Lienhard, G. E. (1973) *Science 180*, 149-154.
Markley, J. L. (1979) *Biological Applications of Magnetic Resonance*, p 397, Academic Press, New York.
Matthews, B. W., Sigler, P. B., Henderson, R., & Blow, D. M. (1967) *Nature (London) 214*, 652.
Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., & Kraut, J. (1975) *J. Biol. Chem. 250*, 7120.
Matthews, D. A., Alden, R. A., Birktoft, J. J., Freeer, S. T., & Kraut, J. (1977) *J. Biol. Chem. 252*, 8875.
Miller, D. D., Horbett, T. A., & Teller, D. C. (1971) *Biochemistry 10*, 4641.
Olah, G. A., & McFarland, C. W. (1971) *J. Org. Chem. 36*, 1374.
Osterbaan, R. A., Kunst, P., & Cohen, J. A. (1955) *Biochem. Biophys. Acta 16*, 299.
Porubcan, M., Westler, W. M., Ibanez, I. B., & Markley, J. L. (1979) *Biochemistry 18*, 4108.
Prado, F. R., Geissner-Prettre, C., Pullman, B., & Daudey, J. P. (1979) *J. Am. Chem. Soc. 101*, 1737-1742.
Reeck, G. R., Nelson, T. B., Prauksteh, J. V., & Mueller, D. D. (1977) *Biochem. Biophys. Res. Commun. 74*, 643.
Robillard, G., & Shulman, R. G. (1974) *J. Mol. Biol. 86*, 519.
Schaffer, Ns. K., May, C. S., & Summerson, W. H. (1953) *J. Biol. Chem. 202*, 67.
Schaffer, N. K., May, C. S., & Summerson, W. Hs. (1954) *J. Biol. Chem. 206*, 201.
Schonbaum, G. R., Zerner, B., & Bender, M. L. (1961) *J. Biol. Chem. 236*, 2930.
Shah, D., Kallick, D., Rowell, R., Chen, R., & Gorenstein, D. G. (1983) *J. Am. Chem. Soc. 105*, 6942.
Sigler, P. B., Blow, D. M., Matthews, B. W., & Henderson, R. (1968) *J. Mol. Biol. 35*, 143.
Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol. 83*, 185.
Taira, K., & Gorenstein, D. G. (1987) *Bull. Chem. Soc. Jpn. 60*, 3625-3632.
Toia, R. F., & Casida, J. E. (1979) *Biochem. Pharmacol. 28*, 3307.
van der Drift, A. C. M., Beck, H. C., Dekker, W. H., Hulst, A. G., & Wils, E. R. (1985) *Biochemistry 24*, 6894-6903.
Vandlen, R. L., & Tulinsky, A. (1973) *Biochemistry 12*, 4193.
Wolfenden, R. L. (1972) *Acc. Chem. Res. 5*, 10.

Multinuclear NMR Studies of the Divalent Metal Binding Site of NADP-Dependent Isocitrate Dehydrogenase from Pig Heart†

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ABSTRACT: The metal activator site of NADP-dependent isocitrate dehydrogenase from pig heart has been probed by using ^{113}Cd and ^{25}Mg NMR as well as manganese paramagnetic relaxation of nuclei in the fast-exchanging ligands α-ketoglutarate and adenosine 2′-monophosphate. Cadmium NMR shows that cadmium, bound to the enzyme in the presence of isocitrate, has a resonance at 9 ppm relative to cadmium perchlorate, while the free Cd–isocitrate complex has a resonance at −23 ppm. Comparison with model compounds and previously studied proteins indicates that cadmium is coordinated with six oxygen ligands. Measurements as a function of cadmium concentration give a dissociation constant of 66 μM and a dissociation rate constant of 1.5×10^4 s^{-1} at pH 7.0. ^{25}Mg NMR demonstrates that the line width of the magnesium resonance is increased upon binding to isocitrate dehydrogenase. A further increase in line width is observed upon addition of isocitrate. Measurement of line widths as a function of temperature reveals that in the binary complex between magnesium and enzyme, exchange is the major contributor to broadening while in the ternary complex containing isocitrate, the intrinsic relaxation in the bound state is also important, suggesting an increase in the dissociation rate constant for magnesium from the ternary complex. Paramagnetic relaxation studies of nuclei of α-ketoglutarate, bicarbonate, and adenosine 2′-monophosphate locate the divalent metal within the active site. The results with adenosine 2′-monophosphate show that atoms in the adenosine moiety of the coenzyme are at least 8 Å from the metal site. In the enzyme complex with α-ketoglutarate, Mn is more than 7 Å from the 1-, 2-, and 5-carbons, but Mn is only 5.0 Å from the carbon of the inhibitor, bicarbonate. A postulated role for Mn is to activate the β-carboxyl of isocitrate that is removed in the enzymatic decarboxylation reaction.

The oxidative decarboxylation of isocitrate to form α-ketoglutarate catalyzed by NADP-dependent isocitrate dehydrogenase from pig heart [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] requires a divalent metal (Villafranca & Colman, 1972; Colman, 1983). This requirement may be satisfied by several divalent metals including manganese, magnesium, or cadmium (Colman, 1972a). The binding of manganese has been extensively

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studied by ultrafiltration (Villafranca & Colman, 1972; Ehrlich & Colman, 1975), nuclear magnetic resonance (Villafranca & Colman, 1972, 1974; Levy & Villafranca, 1977b), and electron spin resonance (Levy & Villafranca, 1977a). The dissociation constant of manganese from the enzyme is reduced more than 20-fold by inclusion of isocitrate. The electron spin resonance spectra indicate that the environment of enzyme-bound manganese changes from a fairly symmetrical one to a distorted one in the presence of isocitrate. Relaxation measurements on water protons indicate that a molecule of water is excluded on going from the binary manganese–enzyme complex to the ternary manganese–isocitrate–enzyme complex. Evidence that isocitrate changes the geometry of enzyme-bound metal is also provided by studies of the quenching of fluorescence of a covalently bound nucleotide analogue by the divalent metal activators Ni^{2+} and Co^{2+} (Bailey & Colman, 1987). A single distance from manganese to the C-4 protons of α-ketoglutarate suggests that manganese may coordinate either the 1-carboxyl and/or the 2-carbonyl group (Villafranca & Colman, 1974).

In the present study, the unique magnetic resonance properties of cadmium, magnesium, and manganese are used to probe the enzyme binding site of isocitrate dehydrogenase. Cadmium-113 chemical shifts are sensitive to changes in the chemical groups and symmetry of the first coordination sphere (Mennitt et al., 1981; Ellis, 1983) with shifts occurring in the range from 800 to −110 ppm relative to cadmium perchlorate in dilute solutions. Measurement of the cadmium shift in the isocitrate dehydrogenase complex should give information on the types of amino acids that constitute the enzyme binding site.

Magnesium-25 has a quadrupole moment and spin $^5/_2$. While the differences in chemical shifts are expected to be small, the relaxation rates should be sensitive to changes in ligand binding (Magnusson & Bothner-By, 1971, Forsén et al., 1981; Drakenberg & Forsén, 1983). Depending upon the magnitude of the exchange rates, the line widths may reflect the electric field gradients at the binding site or the exchange rate. These possibilities may be distinguished by temperature dependence studies. Data are limited for magnesium–enzyme systems, but measurements have been obtained for a prothrombin fragment, factor XIII (Koehler et al., 1987), and calmodulin (Tsai et al., 1987). Magnesium may be the physiological activator, and the present studies demonstrate that, like manganese, magnesium can bind in the presence or absence of isocitrate but the properties of the site are different in the binary and ternary complexes.

Manganese is paramagnetic and can increase the relaxation rates of nuclei on nearby molecules. In order to better define the position of manganese in the complex with enzyme and α-ketoglutarate, the relaxation rates of selectively labeled (C-13) carbons of α-ketoglutarate (Ehrlich & Colman, 1987) have been measured. The position of manganese relative to the nucleotide binding site has been determined from the relaxation behavior of protons and phosphorus atoms of rapidly exchanging nucleotide fragments, adenosine 2′-phosphate, and adenosine 2′,5′-bisphosphate. A portion of this work has been presented in a preliminary version (Ehrlich & Colman, 1986).

Experimental Procedures

Materials. Isocitrate dehydrogenase was purified from pig hearts as described by Bacon et al. (1981). The enzyme was concentrated to 15–30 mg/mL by using Amicon ultrafiltration devices with PM-10 membranes. The enzyme was dialyzed against the indicated buffers containing D_2O. Enzyme concentration was determined by using an extinction coefficient of 1.08 mg^{-1} at 280 nm (Johanson & Colman, 1981) and a subunit molecular weight of 58000 (Colman, 1972b).

Nucleotides were obtained from Sigma. ^{113}Cd (92% enriched) was obtained from Prochem as the free metal and dissolved in HCl. Cadmium nitrate (unenriched) was obtained from Matheson. The concentration of cadmium was measured by titration with ethylenediaminetetraacetic acid (EDTA)[1] and monitoring of the absorbance at 235 nm (Sweetser & Bricker, 1954). The absorbance decreases until an end point, corresponding to equimolar concentrations of cadmium and EDTA, is reached, and the concentration of cadmium is determined from this end point. The concentration was also determined by monitoring the ^{113}Cd NMR spectrum as a function of EDTA concentration: at equimolar concentrations, the amplitude of the resonance due to free cadmium disappears. The concentrations obtained by these methods agree within 3%. ^{25}Mg (95% enriched) was obtained from Merck as the oxide and was dissolved in H_2SO_4. α-Ketoglutarate enriched in ^{13}C was prepared as described by Ehrlich and Colman (1987).

NMR Spectroscopy. ^{113}Cd spectra were obtained at 55.5 MHz in a Bruker WM-250 spectrometer with a 10-mm variable-frequency probe. A saturated solution of cadmium nitrate was used to tune the spectrometer. A sweep width of 10000 Hz was used with 16128 data points. Initial experiments with wider sweep widths were performed to ascertain that no resonances occur outside the range used. The signal-to-noise ratio was enhanced by using routinely an exponential line broadening of 8 Hz. An external reference sample of $Cd(ClO_4)_2$ was used to determine chemical shifts. Positive shifts indicate lower shielding. Sample volumes were 2 mL and include 10% D_2O for spectrometer locking.

^{25}Mg spectra were obtained at 15.3 MHz in a Bruker WM-250 spectrometer using a 15-mm variable-frequency probe. A saturated solution of unenriched $MgSO_4$ was used for initial tuning. The 90° pulse was 100 μs. Typical acquisition and processing values are a sweep width of 10000 Hz with 4096 data points. Because of "ringing" following the pulse, an extra delay of 170 μs was used, or the free induction decay was left-shifted to eliminate the first two points prior to transformation. Enzyme samples were initially dialyzed against buffers containing EDTA to remove extraneous metal and then against the desired buffer. Samples of 4 mL were used with 20% D_2O added as a field lock. Line widths were measured at half-height. In some cases, a Lorentzian was fitted to the spectrum using software provided by Bruker on the Aspect 3000 computer of the AM-250 spectrometer.

Paramagnetic relaxation measurements of ligands were obtained on the Bruker WM-250 spectrometer using a 5-mm probe for ^1H and a 10-mm probe for ^{13}C or ^{31}P. Relaxation times for carbon were long in the absence of metal, and a saturation–recovery pulse sequence was used. A train of 90° pulses was obtained using Bruker software. This was followed by a variable-recovery interval and then a 90° measuring pulse. Proton and phosphorus relaxation measurements were made by using nonselective inversion recovery (Carr–Purcell–Meiboom–Gill) (Meiboom & Gill, 1958). Relaxation times were calculated from peak heights using the nonlinear least-squares fitting routines developed by Dr. Joseph Noggle (F-Curve, LEDS Publishing Co., Research Triangle Park, NC).

The relaxation rate attributable to enzyme-bound manganese was calculated according to the program described by Cohen and Colman (1972) to obtain the concentrations of free

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; 2′-AMP, adenosine 2′-monophosphate.

manganese and of manganese complexes. The relaxation rate attributable to free manganese, measured independently in the same buffer but in the absence of enzyme, was subtracted from the observed relaxation rate to yield the relaxation rate due to enzyme-bound Mn^{2+}. Since the effect of bound manganese is sensitive to errors in calculation of free and bound metal, particularly when the ligand is a good chelator, the calculations for manganese–enzyme–nucleotide complexes were checked in the following way: Samples containing enzyme, manganese, and ligand were dialyzed against a small volume of buffer (typically 1–2 mL) containing the calculated free metal and ligand concentrations. The relaxation rates of the ligand were measured in the dialysis solution and in the dialyzed enzyme sample. The relaxation rate in the dialysis solution was compared with that expected from the calculated concentrations of free ligand and free manganese. Good agreement (within 20%) between relaxation times was obtained, demonstrating the validity of the calculations.

Distances between manganese and atoms on the ligands were obtained by using the Solomon–Bloembergen equations for dipolar relaxation rates (Solomon & Bloembergen, 1956):

$$\frac{1}{T_1} = \frac{C}{r^6}\frac{3\tau_c}{1+\omega^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2} \quad (1)$$

where C depends upon the quantum numbers and gyromagnetic ratios of the nuclei of interest, ω and ω_s are the nuclear and electron spin resonance frequencies, and r is the metal–nucleus distance. Correlation times (τ_c) for enzyme-bound manganese were obtained by using the frequency dependence of water proton relaxation rates and resulting data obtained by Villafranca and Colman (1974). On the basis of this frequency dependence, at the field used for the current measurements the correlation time is dominated by the rotational rate of the enzyme, and the electron spin term does not contribute significantly. Electron spin resonance spectra indicate little change in the electron spin relaxation rate for enzyme–manganese–α-ketoglutarate or enzyme–manganese–nucleotide complexes (Levy & Villafranca, 1977a). The correlation time for the manganese–ligand complex in the absence of enzyme is calculated from the molecular weight dependent rotational correlation time of the manganese–ligand complex using the known correlation time for the $Mn(H_2O)_6$ complex (3×10^{-11} s) (Bloembergen & Morgan, 1961).

Results and Discussion

^{113}Cd NMR. Attempts were made to obtain spectra of enzyme-bound cadmium in the absence of isocitrate. These were not successful because of precipitation of both enzyme and cadmium in these complexes. The absence of such precipitation with magnesium or manganese suggests that cadmium, while an effective activator of enzymatic activity, binds diffeently from the other metals in the absence of ligands. This difference could arise from the preference of cadmium for sulfur over oxygen ligation (Jaffe & Cohn, 1978).

A typical spectrum of ^{113}Cd in the presence of isocitrate is shown in Figure 1A. The chemical shift of ^{113}Cd was measured in the presence of 10–80 mM DL-isocitrate in 0.05 M MOPS (pH 7.0) containing 0.1 M Na_2SO_4 and 10% D_2O. From the isocitrate concentration dependence, a chemical shift for cadmium complexed with isocitrate of −23 ppm [relative to $Cd(ClO_4)_2$] was obtained with a dissociation constant of 2.8 mM. This chemical shift is in the range found for cadmium complexes with acetate (−46 ppm) or acetylacetone (−18 ppm) in which crystallographic evidence shows seven to eight oxygens in the first coordination sphere (Mennitt et al., 1981).

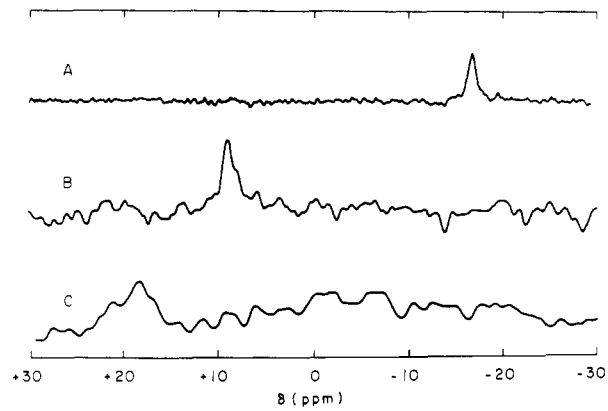

FIGURE 1: ^{113}Cd NMR spectra in the presence of 10 mM DL-isocitrate. The samples are in Na–50 mM MOPS (pH 7.0) containing 100 mM Na_2SO_4. Composition of samples and NMR conditions are as follows: (A) no enzyme, 1.25 mM cadmium, 1000 scans, 8-Hz exponential line broadening; (B) 0.64 mM cadmium, 0.67 mM isocitrate dehydrogenase, 71 000 scans, 32-Hz line broadening used in processing the free induction decay; (C) 0.69 mM cadmium, 0.74 mM isocitrate dehydrogenase, 1 mM phosphoadenosine diphosphoribose, 91 000 scans, 64-Hz line broadening.

As the pH is lowered from 7 to 4.7, the chemical shift at a given concentration of isocitrate increases, approaching that of free cadmium, as would be expected for increased dissociation of the cadmium–isocitrate complex as isocitrate becomes protonated ($pK = 5.7$). The results indicate that most of the cadmium is bound to tribasic isocitrate.

When cadmium is added to a solution containing isocitrate dehydrogenase and 10 mM DL-isocitrate, the cadmium resonance is deshielded relative to the isocitrate–cadmium complex (Figure 1B). The increased line broadening and lower cadmium concentration require an increased number of scans to resolve the signal from the spectrometer noise. A scan of the region from 600 ppm to −130 ppm revealed no other resonances. The chemical shift did not vary by more than 0.2 ppm when the cadmium and isocitrate concentrations were lowered to 0.4 mM, indicating that the measured chemical shift (9.2 ppm) corresponds to that of enzyme-bound cadmium in the presence of isocitrate. The line width of the enzyme-bound resonance is 50 Hz. Spectra obtained in the presence and absence of broad-band proton decoupling were nearly identical, suggesting the absence of nearby protons contributing to relaxation.

The shift observed for enzyme-bound cadmium may be used to indicate the residues close to cadmium by comparison with model compounds or enzymes of known crystallographic structure. The presence of an inner-sphere sulfur may be ruled out since this results in shifts of 400–700 ppm. More than one nitrogen ligand yields shifts of ≥100 ppm (Mennitt et al., 1981; Bailey et al., 1980). A single nitrogen ligand with five oxygen ligands in the Cd–concanavalin A complex gives a shift of 43 ppm (Bailey et al., 1980). Cadmium can bind in configurations with more than six coordinated oxygens. This configuration (typical of calcium binding proteins) gives chemical shifts of about −100 ppm, while shifts in the range from 150 to −60 ppm are characteristic of hexacoordinate cadmium (Ellis, 1983). The chemical shift of 9 ppm observed for Cd in the Cd–isocitrate–isocitrate dehydrogenase complex is characteristic of cadmium bound to six oxygen-containing ligands. A hexacoordinate complex is typical of enzymes using manganese or magnesium. The manganese–isocitrate–enzyme complex has been found to contain a single coordinated water (Villafranca & Colman, 1974) which is undoubtedly coordinated through an oxygen. One or two oxygens may be

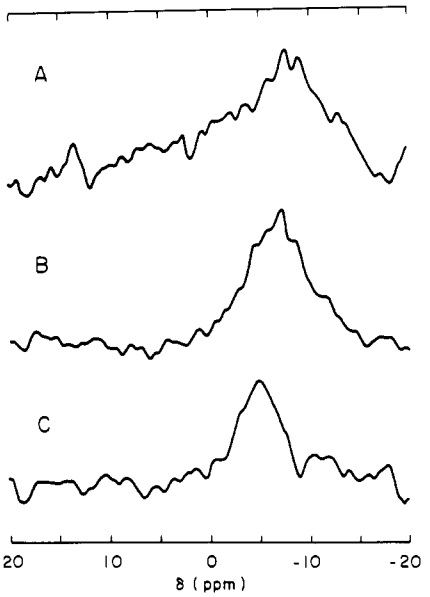

FIGURE 2: Broadening and shifting of ^{113}Cd resonances for Cd:enzyme ratio > 1. All samples contain 10 mM DL-isocitrate. (A) and (B) are in the same buffer (pH 7.0) as used for the spectra in Figure 1. (A) 1.20 mM Cd, 0.36 mM isocitrate dehydrogenase, 39 000 scans, 50-Hz line broadening; (B) 1.57 mM Cd, 0.35 mM enzyme, 94 000 scans, 50-Hz line broadening; (C) 1.09 mM Cd, 0.33 mM isocitrate dehydrogenase, pH 6.3 (Na–50 mM MES), 15 000 scans, 50-Hz line broadening.

supplied by the β-carboxyl of isocitrate. The ^{13}C resonance of this group is perturbed by magnesium binding in the enzyme–magnesium–isocitrate ternary complex (Ehrlich & Colman, 1987). Other oxygens may come from backbone carbonyls or acidic side chains of the enzyme. Carbodiimide modification of a glutamic acid residue in isocitrate dehydrogenase is prevented by metal–isocitrate (Colman, 1973).

Previous experiments have indicated that nucleotides cause changes in the dissociation of isocitrate (Ehrlich & Colman, 1978, 1985). NADPH has been observed to alter the electron spin resonance spectrum of manganese (Levy & Villafranca, 1977a). The cadmium NMR spectrum was measured in the presence of the nucleotide fragment, 2′-phosphoadenosine diphosphoribose, and isocitrate (Figure 1C). The ^{113}Cd resonance is greatly broadened (line width ≃ 300 Hz) and is shifted to 18 ppm. The same broadening and shift are obtained with NADPH. These results demonstrate that an alteration in the cadmium binding site is produced by the binding of nucleotide. The increase in line width could result from an increased cadmium dissociation rate from the enzyme complex or from a heterogeneity in the cadmium binding site(s). An NADPH-induced conformational change in the enzyme–metal–isocitrate complex as postulated by Levy and Villafranca (1977a) from the ESR data may cause the shift in the Cd resonance.

When the concentration of cadmium exceeds that of enzyme, the line width increases greatly, and the resonance shifts upfield (Figure 2A,B). By comparison of the shifts with that of cadmium–isocitrate under the same conditions, the concentrations of free and bound cadmium are estimated.[2] A

[2] To ascertain that the resonance observed includes all of the enzyme-bound cadmium, the areas of cadmium–enzyme–isocitrate peaks were compared with those of free cadmium. Spectra were processed under the same conditions and the peaks cut out and weighed. While the errors are large due to the broad peaks, the upper limit on cadmium not accounted for is 0.5 mol/mol of subunit. Thus, any other cadmium sites, if they exist, must have low affinity for cadmium compared with the isocitrate-dependent site.

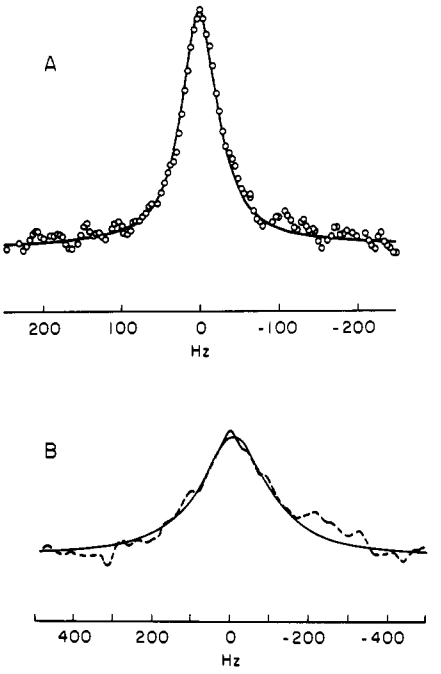

FIGURE 3: ^{25}Mg NMR spectra in the presence of isocitrate dehydrogenase. (A) 6.25 mM Mg with 0.27 mM isocitrate dehydrogenase. The sample is in Na–25 mM MOPS (pH 7.5) containing 25 mM Na_2SO_4 and 10% glycerol. A sweep width of 4000 Hz was used. 12 261 scans were acquired with a delay of 0.25 s between scans. The free induction decay was multiplied by an exponential function with line broadening of 8 Hz. The points result from a Fourier transform using 2048 data points. The solid line is a Lorentzian function fit to the data points with a line width of 56 Hz. (B) 17 mM Mg, 0.19 mM isocitrate dehydrogenase plus 10 mM DL-isocitrate in the same buffer as in (A); 18 000 scans were acquired with no delay between scans. Exponential line broadening was 32 Hz. The solid line is a Lorentzian fit to the Fourier transform (dashed line) using a line width of 209 Hz.

dissociation constant of cadmium from the cadmium–enzyme–isocitrate complex of 66 ± 13 μM is obtained. The line broadening is characteristic of intermediate exchange. A maximal line width is obtained at a cadmium:enzyme ratio of approximately 3 (Figure 2A). Using the intermediate exchange theory presented by Feeney et al. (1979), we calculated a dissociation rate constant of 1.5×10^4 s^{-1}. From $K_D = k_{off}/k_{on}$, the association rate constant k_{on} is 2.3×10^8 M/s. As the pH is lowered from 7 to 6.3, the dissociation constant for metal increases, and the cadmium resonance shifts from −8.1 (Figure 2A) to −5.5 ppm (Figure 2C), closer to that for free metal.

^{25}Mg NMR. The magnesisum resonance in buffered water is narrow (line width about 5 Hz). When enzyme is added, the line width increases as shown in Figure 3A. The line may be reasonably fitted by a Lorentzian. Upon addition of isocitrate to magnesium, the lines broaden, even in the absence of enzyme. When enzyme is added, the line width increases further, as illustrated in Figure 3B.

In order to interpret the line widths measured for magnesium in the various complexes, the temperature dependence of the line widths was measured (Figure 4). The experimental data were analyzed along the lines proposed by Drakenberg et al. (1983). The observed relaxation rates, after subtraction of the contribution of free magnesium (4–5 Hz), may be dependent both upon relaxation in the bound complex (T_2) and on the exchange rate of magnesium from this complex (k_{off}):

$$\pi\Delta\nu = \frac{1}{T_2 + 1/k_{off}} \quad (2)$$

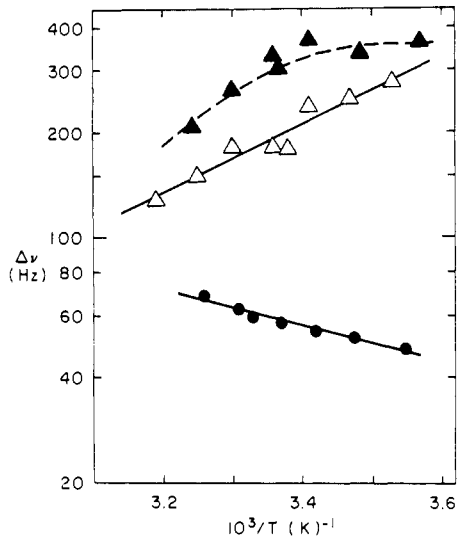

FIGURE 4: Temperature dependence of ^{25}Mg line widths. All measurements were made at pH 7.5 using the conditions of Figure 3. Samples contain 5 mM Mg plus 0.25 mM isocitrate dehydrogenase (●) or 10 mM Mg and 20 mM DL-isocitrate with no enzyme (△) or with the addition of 0.25 mM enzyme (▲). The lines are fits to Arrhenius equations (eq 3 or 5). For the ternary complex (▲---▲), the temperature variations of both τ_c and the dissociation rate are considered. Values of the constants obtained by nonlinear least-squares fitting are given in the text.

If the contribution of relaxation is negligible, the line width depends only on k_{off} which can be written as an Arrhenius equation:

$$k_{off} = \text{const}(e^{-\Delta G_{ex}/RT}) \quad (3)$$

where ΔG_{ex} is the free energy of exchange. For magnesium in the binary complex with isocitrate dehydrogenase, the line width decreases with decreasing temperature [negative slope to the Arrhenius plot in Figure 4 (●)], indicating that exchange between free and bound magnesium dominates the observed broadening. A least-squares fit to the data gives estimates of the free energy of exchange (ΔG_{ex}) of 1.7 kcal/mol with an off rate of 4×10^3 s^{-1} at 298 °C. The maximum possible contribution from relaxation (considering errors in the individual points) is about 20%, which places an upper limit on the value of T_2 of 4×10^{-5} s.

If there is no exchange contribution to relaxation, then the line width depends inversely on the relaxation time. Under the experimental conditions, the relaxation rate ($1/T_2$) is proportional to the rotational correlation time for the magnesium complex (τ_c). If the correlation time obeys an Arrhenius relation:

$$1/\tau_c = \text{const}'(e^{-\Delta G/RT}) \quad (4)$$

then eq 2 becomes

$$\pi\Delta\nu = \text{const}''(e^{\Delta G/RT}) \quad (5)$$

and the Arrhenius plot of the observed data (△) will have a positive slope. The line width of magnesium complexes with isocitrate increases with decreasing temperature (positive slope to the Arrhenius plot in Figure 4). A fit of the data gives a value of 4.4 kcal/mol for the free energy of rotation of the binary complex.

The data for the ternary complex of magnesium with isocitrate and enzyme are more complex as indicated by the curvature of the plot (▲) in Figure 4, and contributions from both exchange and intrinsic relaxation must be included. Quantitative analysis is necessarily limited by the temperature range accessible which in turn is governed by the stability of isocitrate dehydrogenase. As in the analysis of the magnesium NMR data of Tsai et al. (1987), a contribution from the temperature dependence of the dissociation constants is ignored. This will not affect the qualitative results but may alter the values of free energies found. Equations 4–6 are combined to give $\pi\Delta\nu = \text{const}(e^{-\Delta G_{ex}/RT}) + \text{const}''(e^{\Delta G/RT})$, and the corrected experimental data for the contributions of enzyme-bound magnesium to the line widths are fitted to this equation with relaxation and exchange terms. This fit yields estimated values for T_2 (298 °C) = 5.7×10^{-5} s, k_{off}(298 °C) = 5×10^5 s^{-1}, ΔG_{ex} = 23 kcal/mol, and ΔG = 12 kcal/mol. The rate constant for dissociation of magnesium is seen to increase in the ternary complex by about 100-fold compared with the binary complex.

Table I: ^{13}C Relaxation Rates and Calculated Mn–Nucleus Distances for Mn–α-Ketoglutarate and Mn–$NaHCO_3$[a]

compd and functional group	no enzyme		+enzyme	
	$1/fT_1$ (s^{-1})	r (Å)	$1/fT_1$ (s^{-1})	r (Å)
α-keto-glutarate				
1-carboxyl	690 ± 150 (6)	3.9 ± 0.2	59 ± 16 (4)	7.3 ± 0.3
2-carbonyl	570 ± 130 (2)	4.0 ± 0.2	43 ± 5 (4)	7.7 ± 0.2
5-carboxyl	340 ± 27 (4)	4.4 ± 0.1	47 ± 15 (4)	7.6 ± 0.4
bicarbonate				
HCO_3^-	2240 ± 330 (4)	3.2 ± 0.1	600 ± 200 (4)	5.0 ± 0.3

[a] Spin–lattice relaxation rates were measured by the saturation–recovery method. The number in parentheses gives the number of individual measurements used to determine the average and standard deviation. These measurements were made with Mn concentrations from 6 to 32 μM, α-ketoglutarate concentrations from 1.2 to 14.7 mM, and enzyme concentrations of 0.23–0.27 mM. When enzyme is present, the concentration of Mn–α-ketoglutarate is always less than 3% of the concentration of enzyme–Mn–α-ketoglutarate. Observed relaxation rates are corrected for the fraction of the total Mn bound to the enzyme, f. Distances were calculated as described in the text. The error estimates do not include errors arising from the choice of correlation time.

Paramagnetic Relaxation of α-Ketoglutarate. The ^{13}C spin–lattice relaxation times for atoms in α-ketoglutarate are 35, 42, and 31 s for the 1-carboxyl, 2-carbonyl, and 5-carboxyl, respectively. In the presence of manganese, those times are greatly reduced. For example, with 26 μM Mn and 14.7 mM α-ketoglutarate, the relaxation time for the 1-carboxyl is 4.1 s. The relaxation rate ($1/T_1$) for the manganese complex is calculated by using the association constant (39 M^{-1}) given by Kuchel et al. (1980) for this complex. The results are shown in Table I. Using the Solomon–Bloembergen equation for paramagnetic relaxation (eq 1), we can calculate the distances between manganese and carbons. The correlation time may be estimated from the relaxation rate for water protons, which is little changed when waters in the coordination sphere are replaced by α-ketoglutarate or isocitrate (Villafranca & Colman, 1974). The calculated correlation time for the complex is 4.5×10^{-11} s assuming two waters are replaced and using the correlation time for manganese-bound water (3×10^{-11} s). This is similar to that found for manganese coordinated with bicarbonate ion (4.8×10^{-11} s) (Led & Neesgaard, 1987), suggesting independent motion of the carboxyl groups. The distances resulting from use of eq 1 with the above values give distances greater than found for the manganese–citrate complex (3.0 Å) or for the Mn–pyruvate complex (3.5 Å) (Fung et al., 1973). A possible cause of this discrepancy is the interaction of manganese with either carboxyl, resulting in a distribution of complexes with manganese coordinated predominantly with either the 1-carboxyl or the 5-carboxyl. The weak association with α-ketoglutarate compared with isocitrate suggests only a single carboxyl may be involved in the complex.

In the ternary complex of manganese–enzyme and α-ketoglutarate, the relaxation rates attributable to this complex are less than those in the binary complex (Table I). In order to calculate the distance between manganese and α-ketoglutarate in this complex, the correlation time must be known. Colman and Villafranca (1974) have shown that the correlation time for water proton relaxation at high fields (220 MHz for protons) is dominated by the rotational correlation time of the enzyme complex and the electron spin relaxation rate becomes negligible. The measured relaxation rates for α-ketoglutarate are all less than the lower limit on the exchange rate (16.4×10^3 s^{-1}) (Villafranca & Colman, 1974), indicating that they are dominated by paramagnetic dipole relaxation in the bound complex. When the result that the enzyme is a dimer under the current conditions (Bailey & Colman, 1985) is used, this correlation time is 4×10^{-8} s for a spherical complex of 117000 molecular weight. The correlation time may be estimated independently from the ratio of T_1 to T_2 (Duffy & Nowak, 1985). From the ^{13}C line widths of the 1-carboxyl and 5-carboxyl, the ratio is between 50 and 230, yielding a correlation time between 4.1×10^{-8} and 8.8×10^{-8} s, values not greatly different from the value obtained from water proton relaxation measurements. The calculated distances, given in Table I, are greater than 7 Å and show that enzyme-bound manganese is not coordinated with any of the carbons of α-ketoglutarate. Systematic errors resulting from the assumptions of manganese binding constants and correlation times would contribute no more than 0.5 Å to these distances and would not alter the qualitative result. Likewise, the inclusion of a constant, frequency-independent, electron spin relaxation rate equal to that determined by Villafranca and Colman (1974) at 48 MHz (3.5×10^{-7} s^{-1}) would not change the qualitative conclusion that manganese is not proximal to any of the carbons probed. The distances are consistent with the manganese to proton distance (6.3 Å) for protons on carbon-4 found by Colman and Villafranca (1974). The absence of direct interaction between manganese and α-ketoglutarate is also consistent with the failure to observe increased anisotropy in the electron spin resonance spectrum of the ternary complex when compared with the binary metal–enzyme complex (Villafranca & Levy, 1977a).

Paramagnetic Relaxation of Bicarbonate. Bicarbonate ion acts as a competitive inhibitor with respect to CO_2 in the reductive carboxylation reaction of NADP-dependent isocitrate dehydrogenase (Uhr et al., 1974). In the binary complex of metal and bicarbonate, the metal is coordinated with the carboxyl group as indicated by the relaxation rate (Table I). When a correlation time of 4.8×10^{-11} s (Led & Neesgaard, 1987) is used, the calculated distance is 3.2 Å, in close agreement with the distance (3.0 Å) assumed by Led and Neesgaard. For the ternary complex of enzyme–metal and bicarbonate, the measured relaxation rate from data at 40 and 80 mM bicarbonate is 600 s^{-1}. Again, assuming that the appropriate correlation time for the complex is determined by rotation, a distance of 5.0 Å is calculated. The inhibition data of Uhr et al. (1974) imply that the inhibition constant for bicarbonate is about 30 mM. If the binding constant is in the same range, the actual manganese–bicarbonate distance will be within 0.7 Å of the result calculated from the experimental data. The distance found is less than the distance for any of the nuclei of α-ketoglutarate. Bicarbonate could bind in the vicinity of the CO_2 site or the site of the β-carboxyl of enzyme-bound isocitrate. The latter group has been shown to be affected by binding of isocitrate to the magnesium–enzyme complex resulting in a shift in the ^{13}C resonance of this carbon [and not of the α- and γ-carboxyls (Ehrlich & Colman, 1987)]. One or two of the oxygen ligands to Cd deduced from the ^{113}Cd spectrum may arise from the β-carboxyl of isocitrate which is located in close proximity to the bicarbonate site.

Table II: Paramagnetic Relaxation of Nuclei of 2′-AMP and Distances from Mn to These Nuclei in Complexes with or without Isocitrate Dehydrogenase[a]

complex	nucleus	$1/fT_1$ (s^{-1})	r (Å)
Mn–2′-AMP	8-H	2.5×10^4	4.0 ± 0.2
	2-H	1.0×10^4	4.7
	1′-H	1.4×10^4	4.4
	P	2.9×10^4	2.9
Mn–enzyme–2′-AMP	8-H	326	8.3 ± 0.6
	2-H	166	9.2
	1′-H	<50	>11
	P	<10	>9.8
Mn–enzyme–2′-AMP–isocitrate	8-H	316	8.3 ± 0.4
	2-H	217	8.8
	1′-H	<40	>11
	P	<8	>10

[a] Relaxation rates were measured by the inversion–recovery method. Samples contain, where indicated, 3.8–11.3 mM 2′-AMP, 0.21–0.25 mM isocitrate dehydrogenase, 10 mM DL-isocitrate, and 10–50 μM Mn. Relaxation times were corrected by multiplication by f, the ratio of enzyme-bound metal to 2′-AMP. Distances were calculated by using assumptions for the correlation times discussed in the text.

Paramagnetic Relaxation of Nuclei in the Coenzyme Site. For distance measurements, the ligand should be in fast exchange with the metal–enzyme complex. ^{31}P measurements have shown that the coenzymes $NADP^+$ and NADPH are in slow exchange (Mas & Colman, 1984). 2′-AMP has been shown to be competitive inhibitor with respect to $NADP^+$ and in fast exchange with the enzyme (Ehrlich & Colman, 1978, 1985). Relaxation rates for this compound are given in Table II. For the binary complex with metal, distances were calcuated by using a rotational correlation time for the complex of 1.2×10^{-10} s. The calculated Mn–P distance of 2.9 Å is consistent with the distances (2.8–3.1 Å) found for other phosphate-containing compounds (Duffy & Nowak, 1985; Sloan & Mildvan, 1976). The results indicate possible interaction of metal with the N-7 region of the adenine ring as evidence by the distance from Mn to the H-8 proton. In the Mn–ATP complex, a distance of 4.5 Å was found by Sloan and Mildvan (1976). When isocitrate is added to the binary complex, the distances found are the same within 0.2 Å when correction is made for the complexation of metal by isocitrate.

The measured relaxation rates decrease when Mn and 2′-AMP are added to 0.21–0.4 mM enzyme. The rates were corrected by calculating the distribution of manganese using published dissociation constants (association constant 240 M^{-1} for Mn–2′-AMP) [Sillen & Martell (1971) and other references tabulated by Villafranca and Colman (1972)]. The concentration of 2′-AMP was assumed to be almost saturating based upon the inhibition constant of 0.6 mM (Ehrlich & Colman, 1978) or the dissociation constant of 2.8 mM (Ehrlich & Colman, 1985). The presence of isocitrate tightens the binding of manganese to the enzyme (Villafranca & Colman, 1972), and the correction becomes small. In the quaternary complex, the measured relaxation times (3.6 s for ^{31}P at 50 μM Mn) are actually little different from that of 2′-AMP in the absence of the paramagnetic metal (4.4 s). The corrections for relaxation by free metal were checked by dialyzing the enzyme complex against buffer or filtering the enzyme solution and measuring the relaxation rate of 2′-AMP in the dialysate or filtrate. Errors in the calculated relaxation rates beyond those in the experimental relaxation times are less than 50%, leading to corresponding errors in distances of less than 10%.

In all cases, the measured relaxation rates are less than the lower limit on the dissociation rate ($\simeq 3000\ s^{-1}$) that may be inferred from the line broadening and chemical shift measurements of Ehrlich and Colman (1985), indicating that exchange may be neglected. All the calculated distances are larger than 8 Å (the precision of the NMR relaxation method deteriorates in this range), indicating that the metal site is far from the 2′-AMP moiety of bound coenzyme. The results are consistent with the ^{31}P experiments that showed that metal does not alter the resonances of the phosphates of bound coenzymes (Mas & Colman, 1984).

Similar results were obtained with adenosine 2′,5′-bisphosphate although the phosphates cannot be resolved and a Mn–P distance was not measured. Proton relaxation rates indicate that, in the binary Mn–nucleotide complex, Mn is considerably closer to the 8-proton (3.6 Å) than the 1′-proton, suggesting that primary coordination is through the 5′-phosphate and the N-7 nitrogen. For complexes with Mn, adenosine 2′,5′-bisphosphate, and enzyme, the proton relaxation times were the same within the experimental error for the solution containing enzyme and the ultrafiltrate from which enzyme was removed. Thus, as with 2′-AMP, distances to all protons in the ternary complex must be greater than 7 Å, indicating a lack of direct interaction of manganese with groups in the adenosine nucleotide moiety.

Conclusions. Cd NMR has yielded specific information on coordination in the ternary metal–isocitrate–isocitrate dehydrogenase complex. The chemical shift is in the range that is clearly characteristic of hexacoordinate oxygen ligation. This coordination is typical of magnesium, while cadmium may mimic a variety of metals by forming different coordination complexes. The exchange rate of cadmium from this complex is slower than that of magnesium, consistent with the tighter binding of cadmium. Cadmium NMR has indicated that the cadmium–isocitrate complex can still bind to the enzyme in the presence of nucleotides. However, the binding site is altered as evidenced by a shift and broadening of the cadmium resonance.

Multinuclear NMR spectroscpy has yielded complementary information about the metal site of NADP-dependent isocitrate dehydrogenase. The line width of magnesium bound to the enzyme increases upon formation of the ternary complex with isocitrate. The temperature dependence of the line widths indicates the increase arises from an increase in the rate constant for dissociation.

Paramagnetic relaxation by enzyme-bound manganese has distinguished between the conjectures that Mn is not involved in an inner-sphere complex with α-ketoglutarate (Levy & Villafranca, 1977a,b) and that Mn may be coordinated with the carbonyl of enzyme-bound α-ketoglutarate (Villafranca & Colman, 1974). Carbonyl coordination was indicated as consistent with the lack of a carbon isotope effect, but the isotope effect studies do not force this conclusion since they merely indicate that the chemical steps are not rate limiting (O'Leary & Limburg, 1977). Clearly, in the ternary complex, there is no coordination of metal and α-ketoglutarate since all distances are greater than 6 Å. The distance measured by proton NMR (Villafranca & Colman, 1974) was consistent with both possibilities.

Relaxation measurements have established that Mn is distal from the adenosine moiety of the coenzyme. The measured distances between Mn and the 2′-phosphate and adenosine ring protons (8–10 Å) for the quaternary complex containing nucleotide, metal, isocitrate, and enzyme are consistent with the metal–adenine distance measured by fluorescent energy transfer to a covalently bound coenzyme affinity label (Bailey & Colman, 1987) (8 Å). The distances measured in the absence of isocitrate are approximately the same, in contrast to the fluorescence results ($r > 12$ Å). This could arise from the greater errors in allowing for contributions from free metal in these experiments or to differences in binding of Mn to the enzyme compared with Ni and Co used for the fluorescence experiments. The fluorescence energy transfer is more sensitive in this distance range than the NMR method. It is not known whether the distances measured by NMR are between metal and nucleotide on the same subunit of the dimer or on different subunits. From the kinetics of the fluorescently labeled enzyme, it was suggested that the distances are between metal on one subunit and nucleotide near the catalytic site of the other subunit (Bailey & Colman, 1987).

The evidence suggests that only bicarbonate is close to enzyme-bound Mn. Since bicarbonate is a competitive inhibitor with respect to CO_2, the possibility arises that Mn plays a role in the activation of the carboxylate group removed during the isocitrate dehydrogenase reaction. Coordination of magnesium with this group is consistent with the large shift in the carbon resonance by metal in the enzyme–magnesium–isocitrate complex (Ehrlich & Colman, 1987).

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Registry No. Cd, 7440-43-9; Mg, 7439-95-4; Mn, 7439-96-5; NADP-dependent isocitrate dehydrogenase, 9028-48-2; DL-isocitrate, 18979-21-0; adenosine 2′-monophosphate, 130-49-4; α-ketoglutarate, 328-50-7; bicarbonate, 71-52-3.

References

Bacon, C. R., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 6593–6599.

Bailey, D. B., Ellis, P. D., & Fee, J. A. (1980) *Biochemistry 19*, 591–596.

Bailey, J. M., & Colman, R. F. (1985) *Biochemistry 24*, 5367–5377.

Bailey, J. M., & Colman, R. F. (1987) *Biochemistry 26*, 4893–4900.

Bloembergen, N. J., & Morgan, L. O. (1961) *J. Chem. Phys. 34*, 842–850.

Cohen, P. F., & Colman, R. F. (1972) *Biochemistry 11*, 1501–1508.

Colman, R. F. (1972a) *J. Biol. Chem. 247*, 215–223.

Colman, R. F. (1972b) *J. Biol. Chem. 247*, 6727–6729.

Colman, R. F. (1973) *J. Biol. Chem. 248*, 8137–8140.

Colman, R. F. (1983) *Pept. Protein Rev. 1*, 41–69.

Drakenberg, T., & Forsén, S. (1983) in *The Multinuclear Approach To NMR Spectroscopy* (Lambert, J. B., & Riddell, F. G., Eds.) pp 309–328, D. Reidel, Dordrecht.

Drakenberg, T., Forsén S., & Lilja, H. (1983) *J. Magn. Reson. 53*, 412–422.

Duffy, T. H., & Nowak, T. (1985) *Biochemistry 24*, 1152–1160.

Ehrlich, R. S., & Colman, R. F. (1975) *Biochemistry 14*, 5008–5016.

Ehrlich, R. S., & Colman, R. F. (1978) *Eur. J. Biochem. 89*, 575–587.

Ehrlich, R. S., & Colman, R. F. (1985) *Biochemistry 24*, 5378–5387.

Ehrlich, R. S., & Colman, R. F. (1986) *Fed. Proc. 45*, 1648.

Ehrlich, R. S., & Colman, R. F. (1987) *Biochemistry 26*, 3461–3466.

Ellis, P. D. (1983) *Science 221*, 1141–1146.

Feeney, J., Batchelor, J. G., Albrand, J. P., & Roberts, G. C. K. (1979) *J. Magn. Reson. 33*, 519–529.
Forsén, S., Andersson, T., Drakenberg, T., Thulin, E., & Wieloch, T. (1981) in *Advances in Solution Chemistry* (Betini, I., Lunazzi, L., & Dei, A., Eds.) pp 191–208, Plenum, New York.
Fung, C. H., Mildvan, A. S., Allerhand, A., Komoroski, R., & Scrutton, M. C. (1973) *Biochemistry 12*, 620–629.
Jaffe, E. K., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4823–4825.
Johanson, R. A., & Colman, R. F. (1981) *Arch. Biochem. Biophys. 207*, 9–20, 21–31.
Koehler, L. S., Jarnagin, F., Hiskey, R. G., Pederson, L. G., & Koehler, K. A. (1987) *J. Inorg. Biochem. 29*, 153–164.
Kuchel, P. W., Reynolds, C. H., & Dalziel, K. (1980) *Eur. J. Biochem. 110*, 465–473.
Led, J. J., & Neesgaard, E. (1987) *Biochemistry 26*, 183–192.
Levy, R. S., & Villafranca, J. J. (1977a) *Biochemistry 16*, 3293–3301.
Levy, R. S., & Villafranca, J. J. (1977b) *Biochemistry 16*, 3301–3309.
Magnusson, J. A., & Bothner-By, A. A. (1971) in *Magnetic Resonances in Biological Research* (Franconi, C., Ed.) pp 365–376, Gordon and Breach, New York.
Mas, M. T., & Colman, R. F. (1985) *Biochemistry 24*, 1634–1646.
Meiboom, S., & Gill, D. (1958) *Rev. Sci. Instrum. 29*, 688–691.
Mennitt, P. G., Shatlock, M. P., Bartuska, V. J., & Maciel, G. E. (1981) *J. Phys. Chem. 85*, 2087–2091.
O'Leary, M. H., & Limburg, J. A. (1977) *Biochemistry 16*, 1129–1135.
Sillen, L. G., & Martell, A. E. (1971) *Spec. Publ.—R. Soc. Chem. No. 25*, 643.
Sloan, D. L., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2412–2420.
Solomon, I., & Bloembergen, N. J. (1956) *J. Chem. Phys. 25*, 261–266.
Sweetser, P. D., & Bricker, C. E. (1954) *Anal. Chem. 26*, 195–197.
Tsai, M.-D., Drakenberg, T., Thulin, E., & Forsén, S. (1987) *Biochemistry 26*, 3635–3643.
Uhr, M. L., Thompson, V. W., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2920–2927.
Villafranca, J. J., & Colman, R. F. (1972) *J. Biol. Chem. 247*, 209–214.
Villafranca, J. J.; & Colman, R. F. (1974) *Biochemistry 13*, 1152–1160.

Dicyclohexylcarbodiimide Cross-Links Two Conserved Residues, Asp-184 and Lys-72, at the Active Site of the Catalytic Subunit of cAMP-Dependent Protein Kinase†

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ABSTRACT: In the absence of MgATP, the catalytic subunit of cAMP-dependent protein kinase is irreversibly inhibited by the hydrophobic carbodiimide dicyclohexylcarbodiimide, and this inhibition is most likely due to the formation of a cross-link between a carboxyl group and a lysine residue in the active site (Toner-Webb & Taylor, 1987). In order to identify these cross-linked residues, the catalytic subunit was modified by dicyclohexylcarbodiimide and then treated with acetic anhydride and digested with trypsin. The resulting peptides were resolved by high-performance liquid chromatography. One major absorbing tryptic peptide and one smaller peptide consistently and reproducibly showed a decrease in absorbance after the catalytic subunit had been treated with DCCD. These peptides correspond to residues 166–190 and 57–93, respectively. A unique peptide was isolated from the modified catalytic subunit, and the sequence of this peptide established that the cross-linking occurred between Asp-184 and Lys-72. The cross-linking of these two residues, which were both identified previously as essential residues, confirms the likelihood that each plays a role in the functioning of this enzyme. The fact that Asp-184 and Lys-72 appear to be invariant in all protein kinases further supports the hypothesis that these two residues, located close to one another at the active site of the enzyme, play essential roles in catalysis.

The catalytic (C) subunit of cAMP-dependent protein kinase is a monomeric protein with a molecular weight of 40 900 (Shoji et al., 1983). It is one of the simplest protein kinases, because the major regulatory element is part of a separate subunit that dissociates in the presence of cAMP. In its dissociated, monomeric state, the C-subunit is fully active and transfers phosphate from the γ-position of ATP to the hydroxyl group of either a Ser or a Thr residue on a protein substrate. The phosphorylated residue on the substrate is preceded by two basic amino acids, usually arginines, that are required for recognition (Feramisco et al., 1980; Kemp et al., 1977). There is usually a single intervening residue between the basic residues and the phosphorylated amino acid [for a review, see Bramson et al. (1984)].

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