Nuclear Magnetic Resonance Study of Ligand Binding to Mn-Aspartate Transcarbamylase†

S. Fan, L. W. Harrison,‡ and Gordon G. Hammes*

ABSTRACT: Aspartate transcarbamylase from *Escherichia coli* has been prepared with up to four of the zinc ions replaced by manganese, and the effect of this substitution on the proton nuclear magnetic resonance properties of succinate bound to the catalytic site and of cytidine 5′-triphosphate bound to the regulatory site has been determined. The specific activity and allosteric properties of the Mn-substituted enzyme are essentially identical with those of the native enzyme. The longitudinal relaxation time, T_1, of the succinate protons is shortened by the native enzyme and is shortened further by the Mn-substituted enzyme at both 100 and 220 MHz in D_2O solutions of 0.02 *M* imidazole chloride (pH 7.0), 10^{-3} *M* β-mercaptoethanol, 0.2 m*M* ethylenediaminetetraacetic acid, and 2.5 m*M* carbamyl phosphate over a temperature range of 5 to 35°. Under the same conditions, the transverse relaxation time, T_2, of the succinate protons at 90 MHz is shortened to the same extent by native and Mn-substituted enzyme. The temperature dependence of the relaxation times indicates that the shortening of the transverse relaxation time is determined by the lifetime of bound succinate, whereas the further shortening of the longitudinal relaxation time by the Mn-substituted enzyme is due to dipolar relaxation, i.e. to the interaction between Mn and the succinate protons. The distance between the Mn and the protons of succinate bound to the enzyme can be calculated from the relaxation time measurements and is 15.3 Å. The dipolar interaction correlation time, which is needed for the calculation of this distance, was found to be 3.5×10^{-9} sec from the frequency dependence of T_1. The transverse relaxation time of the C-6 proton of CTP is shortened to the same extent by both the native and Mn-substituted enzyme in D_2O solutions of 0.02 *M* imidazole chloride (pH 7.0), 10^{-3} *M* β-mercaptoethanol, 0.2 m*M* ethylenediaminetetraacetic acid, and 2.5 m*M* carbamyl phosphate over the temperature 5–30°. Since the temperature dependence of the relaxation time indicates the relaxation is not exchange limited, the manganese must be too distant from the bound CTP for an appreciable interaction to occur. This requires that the manganese be greater than 20 Å from the CTP. These results are used together with other available structural data to construct a schematic model for aspartate transcarbamylase.

Aspartate transcarbamylase is an allosteric enzyme regulated by nucleotides. This enzyme has been extensively studied with equilibrium, kinetic, and structural techniques (cf. Jacobson and Stark, 1973; Hammes and Wu, 1974). The native enzyme contains two trimeric catalytic subunits and three dimeric regulatory subunits (Gerhart and Schachman, 1965, 1968; Weber, 1968; Rosenbusch and Weber, 1971), with six catalytic sites (Hammes et al., 1970; Rosenbusch and Griffin, 1973) and six regulatory sites (Hammes et al., 1970; Winlund and Chamberlin, 1970; Cook, 1972; Matsumoto and Hammes, 1973; Gray et al., 1973). The overall shape of the enzyme has been established by X-ray diffraction (Evans et al., 1972, 1973, 1974; Warren et al., 1973) and electron microscopy (Richards and Williams, 1972). The location of the catalytic and regulatory sites and the sulfhydryl and tryptophan residues of the catalytic subunit with respect to the overall structure has been determined with fluorescence energy transfer measurements (Matsumoto and Hammes, 1975).

Native aspartate transcarbamylase contains six zinc atoms per enzyme molecule which are located on the regulatory subunit, probably near the interface of the catalytic and regulatory subunits (Rosenbusch and Weber, 1971; Cohlberg et al., 1972; Nelbach et al., 1972). The spatial relationship between the metal ion and other parts of the molecule could be effectively probed if the zinc could be replaced with a paramagnetic species (Mildvan and Cohn, 1970; Dwek, 1973). In this work, manganese was substituted for some of the zinc atoms, and the effect of this substitution on the nuclear magnetic resonance (NMR) relaxation times of the methylene protons of succinate bound to the catalytic site and the C-6 proton of CTP bound to the regulatory site was measured. The results obtained permit the calculation of the distance between the manganese and succinate bound to the enzyme and permit a lower bound to be established for the distance between manganese and CTP bound to the enzyme.

Experimental Section

Chemicals. The D_2O (99.8%) solutions of succinic acid (Sigma Chemical Co.) were titrated with concentrated KOD (prepared by dissolving KOH in D_2O) to pH 6.6 (uncorrected meter reading). The dilithium carbamyl phosphate (Sigma Chemical Co.) was purified by precipitation from aqueous solution with cold ethanol (Gerhart and Pardee, 1962); carbamyl phosphate solutions were prepared immediately before use and kept on ice. Imidazole was recrystallized twice from benzene. Atomic absorption standard manganese solution was obtained from Fisher Scientific Co. All other chemicals used were the best available commercial grades.

Preparation of Mn-Aspartate Transcarbamylase. Aspartate transcarbamylase was purified from the mutant strain of *Escherichia coli* grown by the New England Enzyme Center (Gerhart and Holoubek, 1967). The enzyme was

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‡ National Institutes of Health Postdoctoral Fellow, 1971–1973.

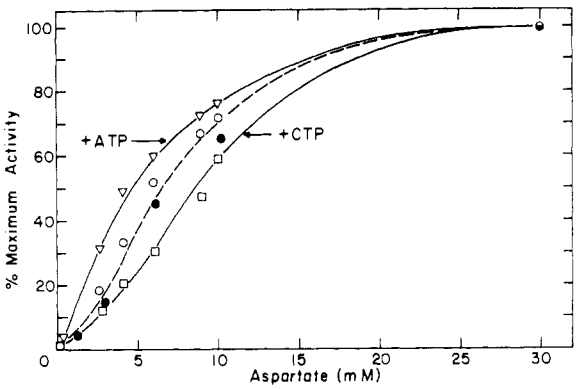

FIGURE 1: A plot of the percent maximum activity vs. the aspartate concentration for the native (●) and Mn-substituted enzymes (○) and for the Mn-substituted enzyme in the presence of 4×10^{-2} *M* CTP (□) and 1.4×10^{-2} *M* ATP (▽). The assay mixture contained 0.1 *M* imidazole (pH 7.0) and 4 m*M* carbamyl phosphate, and the assays were carried out at 23°.

dissociated into catalytic and regulatory subunits (Schachman, 1972), and zinc acetate was added to 2 m*M* to stabilize the regulatory subunit (Nelbach et al., 1972). The metal free regulatory subunit was prepared by dialyzing 50 mg of the regulatory subunit in 4 ml against 2 l. of 0.04 *M* potassium phosphate (pH 7.0), 10^{-2} *M* EDTA, and 10^{-2} *M* β-mercaptoethanol at 4° for approximately 36 hr. Three buffer changes were made during the dialysis. The metal free regulatory subunit then was dialyzed against 0.05 *M* imidazole chloride (pH 7.0) and 10^{-2} *M* β-mercaptoethanol at 4°. A similar dialysis sequence was carried out with approximately 100 mg of the catalytic subunit. The Mn-substituted enzyme was obtained by mixing the dialyzed preparations of catalytic and regulatory subunits (with a one-third mole excess of regulatory subunit) and then dialyzing the mixture against 0.05 *M* imidazole acetate (pH 7.0), 1×10^{-3} *M* atomic absorption standard Mn^{2+}, and 5×10^{-3} *M* β-mercaptoethanol for 15 hr at 4°. During the above dialysis, nitrogen was bubbled into the buffer solutions to minimize the oxidation of protein sulfhydryl groups. After dialysis the enzyme solution was centrifuged to remove precipitated denatured material, and the supernatant was purified by passing it twice through a G200 Sephadex column (2.8 cm i.d. × 16 cm) at 4° using 0.05 *M* imidazole acetate (pH 7.0)–5×10^{-3} *M* β-mercaptoethanol as the elution buffer. The reconstituted Mn-substituted enzyme eluted just after the void volume. The column fractions were identified by polyacrylamide gel electrophoresis as described below. Great care was taken to minimize metal contamination in the preparation described above: all buffers were extracted with 0.001% dithizone in CCl_4 at least three times (Thiers, 1957); the glassware was soaked in 50% nitric acid and rinsed with deionized water many times; plastic labware, which was used as containers for all regulatory subunit solutions, was soaked in 20 m*M* EDTA before use.

The enzyme activity was determined either by the pH-Stat method (Wu and Hammes, 1973) or by a colorimetric assay (method 1; Prescott and Jones, 1969).

Analytical Methods. The concentrations of native and Mn-substituted enzyme were determined by the method of Lowry et al. (1951) using crystallized bovine serum albumin as a standard.

The amount of Mn and Zn in aspartate transcarbamylase was determined by atomic absorption (Suzuki and Wacker, 1974). The Mn-substituted enzyme contains both Zn and Mn, with 2–4 Mn atoms per mole of enzyme (mol wt 310,000), depending on the particular preparation. The enzyme used for all of the NMR measurements reported here contained 3 Mn atoms and 3 Zn atoms per enzyme molecule.

Polyacrylamide gel electrophoresis of the Sephadex column fractions was carried out in 0.25 *M* Tris and 0.2 *M* glycine (pH 8.3), and the gels were stained with Coomassie Blue (Davis, 1964). A good resolution of the holoenzyme and catalytic and regulatory subunits was obtained.

Nuclear Magnetic Resonance Measurements. Longitudinal relaxation times (T_1) of the succinate resonance in the presence and absence of enzyme were measured on Varian XL100 and HR220 spectrometers equipped with a Fourier transform accessory. The homogeneity spoil principle (McDonald and Leigh, 1973) was used to determine T_1. The precision of the T_1 determinations was about ±5% with the HR220 instrument and about ±10% with the XL100 instrument. Measurements of the transverse relaxation time (T_2) of succinate and CTP resonances in the presence and absence of enzyme were made on a Brucker X-90 spectrometer. In the case of CTP, only the relaxation times of the doublet resonance lines of the C-6 proton of CTP were studied in detail. The HDO peak in the sample was used as an internal standard and as a locking signal for the Brucker X-90. The values of T_2 were measured from the spectral line width at one-half of the resonance peak height, $\Delta\nu_{1/2}$, using the relationship $T_2 = (\pi\Delta\nu_{1/2})^{-1}$. Both T_1 and T_2 were measured as a function of temperature. The actual probe temperatures were determined either from the chemical shift of the resonances of methanol or from a thermocouple placed in the probe prior to the relaxation time measurement. The uncertainty in the temperature is about ±1°. In all cases, the nuclear magnetic resonance measurements were carried out in D_2O solutions of 0.02 *M* imidazole chloride (pH 7.0, uncorrected pH meter reading), 10^{-3} *M* β-mercaptoethanol, 0.2 m*M* EDTA, and 2.5 m*M* carbamyl phosphate.

Results

The native enzyme and the enzyme containing Mn have the same mobility on polyacrylamide gel electrophoresis in 0.025 *M* Tris–0.2 *M* glycine (pH 8.3). In both cases weakly stained bands are found just above and below the main band. In addition, the Mn-substituted enzyme is contaminated by a small amount of regulatory subunit (less than 10% as judged by spectrophotometric scanning of the gel). The use of excess regulatory subunit in the reconstitution process prevents the formation of regulatory subunit deficient enzyme species. The Mn-substituted enzyme is much less stable than the native enzyme. On standing at 4° in 0.04 *M* potassium phosphate (pH 7.0) at a concentration of about 25 mg/ml, the Mn-substituted enzyme begins to precipitate after about 1 week. It is even less stable in buffers other than phosphate. The specific activity of the soluble enzyme remaining increases with time, and the Mn content of the enzyme decreases while the Zn content increases. A disperse band in the polyacrylamide electrophoresis gels also appears around the positions expected for the subunits; the intensity of this band increases with time. These results suggest the enzyme is dissociating into subunits and that some Mn-enzyme species are precipitated.

The specific activities of the native and Mn-substituted enzymes are essentially identical (about 85 units/(mg min)), and as shown in Figure 1 the dependence of the ini-

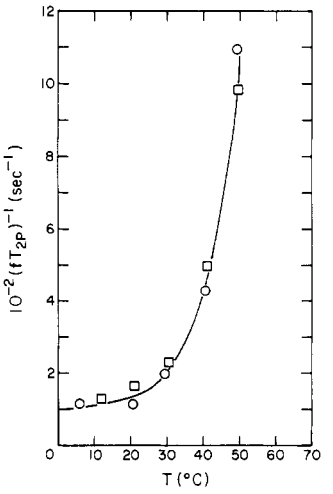

FIGURE 2: A plot of the reciprocal of the transverse relaxation time of the succinate protons defined by eq 1, T_{2p}, times the fraction of succinate bound to the enzyme, f, vs. the temperature for native enzyme (○) and Mn-substituted enzyme (□) at 90 MHz in D_2O solutions of 0.02 M imidazole chloride (pH 7.0, uncorrected pH meter reading), 10^{-3} M β-mercaptoethanol, 0.2 mM EDTA, and 2.5 mM carbamyl phosphate. The enzyme concentration was 4.4×10^{-5} M and the succinate concentration was 10^{-2} M.

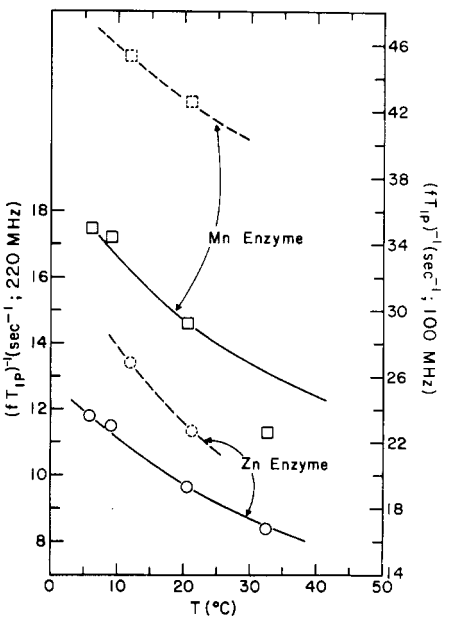

FIGURE 3: A plot of the reciprocal of the longitudinal relaxation time of the succinate protons defined by eq 1, T_{1p}, times the fraction of succinate bound to the enzyme, f, vs. the temperature for native enzyme (○, 220 MHz; ◌, 100 MHz) and Mn-substituted enzyme (□, 220 MHz; ⬚, 100 MHz) in D_2O solutions of 0.02 M imidazole chloride (pH 7.0, uncorrected pH meter reading), 10^{-3} M β-mercaptoethanol, 0.2 mM EDTA, and 2.5 mM carbamyl phosphate. The enzyme concentration was 4.4×10^{-5} M and the succinate concentration was 2.5×10^{-2} M at 100 MHz and 1.0×10^{-2} M at 220 MHz.

tial steady-state velocity on the aspartate concentration also is similar for both enzymes. Moreover, CTP increases the sigmoidicity of the initial velocity-aspartate isotherm of the Mn-substituted enzyme while ATP decreases it (Figure 1). The initial velocities in Figure 1 were measured colorimetrically (method 1; Prescott and Jones, 1969) in 0.1 M imidazole acetate (pH 7.0). The assay mixture was incubated at 23° for 20 min prior to colorimetric analysis. Thus, the Mn-substituted enzyme is a good catalyst and retains an allosteric response to CTP and ATP.

Succinate is an aspartate analog which binds tightly to aspartate transcarbamylase in the presence of carbamyl phosphate (Changeux et al., 1968; Sykes et al., 1970). The temperature dependence of the longitudinal (T_1) and transverse (T_2) relaxation times of the succinate resonance has been measured in the presence of native and Mn-substituted enzyme. The succinate concentration was approximately 10^{-2} M and the enzyme concentration was approximately 3×10^{-5} M in 0.02 M imidazole (pH 7.0), 10^{-2} M β-mercaptoethanol, 0.2 mM EDTA, and 2.5 mM carbamyl phosphate. Most of the measurements of T_1 were made at 220 MHz, but some measurements also were made at 100 MHz. The measurements of T_2 were carried out at 90 MHz. If succinate is assumed to exist in two environments, bound and unbound, the reciprocal relaxation times for succinate protons interacting with a paramagnetic species can be written as (Dwek, 1973):

$$\frac{1}{T_{1,2O}} = \frac{1}{T_{1,2}} + \frac{f}{T_{1,2M} + \tau_h}$$

or

$$\frac{1}{f}\left(\frac{1}{T_{1,2O}} - \frac{1}{T_{1,2}}\right) = \frac{1}{T_{1,2M} + \tau_h} \equiv \frac{1}{fT_{1,2p}} \qquad (1)$$

where $T_{1,2O}$ is the observed longitudinal or transverse relaxation time, $T_{1,2}$ and $T_{1,2M}$ are the corresponding relaxation times for free and bound ligand, respectively, τ_h is the residence time of succinate on the enzyme, and f is the fraction of succinate bound to the enzyme. At the concentrations of succinate and enzyme used, the enzyme is saturated approximately 98% with succinate (Changeux and Rubin, 1968). Two limiting cases can be delineated which lead to a simplification of eq 1: fast exchange and slow exchange of succinate between the two environments. In the former case T_{1M} or $T_{2M} \gg \tau_h$, while in the latter case $\tau_h \gg T_{1M}$ or T_{2M}. (The meaning of fast and slow exchange must be defined separately for each relaxation time.) For fast exchange, $(fT_{2p})^{-1}$ should decrease as the temperature increases, while for slow exchange it should increase with increasing temperature (Swift and Connick, 1962; Luz and Meiboom, 1964). A plot of $(fT_{2p})^{-1}$ vs. temperature for the methylene protons of succinate in the presence of native and Mn-substituted enzyme is presented in Figure 2. The temperature dependence of this relaxation time indicates it is dominated by τ_h although some influence of T_{2M} can be seen at lower temperatures. Since essentially identical values of $(fT_{2p})^{-1}$ are obtained with the native and Mn-substituted enzymes, it can be concluded that the rate of succinate exchange is essentially identical in both cases.

In Figure 3, a plot of $(fT_{1p})^{-1}$ vs. temperature for the methylene protons of succinate in the presence of native and Mn-substituted enzyme is shown at 220 and 100 MHz. At temperatures greater than 35°, the Mn-substituted enzyme is very unstable and begins to precipitate; therefore the relaxation time measurement at 37° is not reliable. In this case, the temperature dependence of the relaxation time indicates the fast exchange limit is occurring. This is also consistent with the finding that $(fT_{1p})^{-1} \ll (fT_{2p})^{-1}$. The difference in the relaxation times of the Zn- and Mn-substituted enzymes can be attributed to the dipolar interaction of Mn with the methylene protons. The longitudinal relaxation rate of the bound substrate protons due to a dipolar interaction with a paramagnetic species at a distance r is given by eq 2 (Solomon, 1955). In this equation S is the electron spin

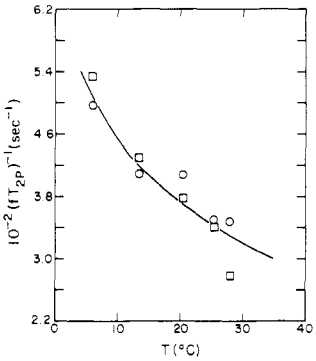

FIGURE 4: A plot of the reciprocal of the transverse relaxation time of the C-6 proton of CTP defined by eq 1, T_{2p}, multiplied by the fraction of bound CTP, f, vs. the temperature for native enzyme (○) and Mn-substituted enzyme (□) at 90 MHz in D_2O solutions of 0.02 M imidazole chloride (pH 7.0, uncorrected pH meter reading), 10^{-3} M β-mercaptoethanol, 0.2 mM EDTA, and 2.5 mM carbamyl phosphate. The enzyme concentration was 3×10^{-5} M and the CTP concentration was 10^{-2} M.

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)g^2\beta^2g_N{}^2\beta_N{}^2}{r^6} \times \left[\frac{3\tau_c}{1+\omega_I{}^2\tau_c{}^2} + \frac{7\tau_c}{1+\omega_s{}^2\tau_c{}^2}\right] \quad (2)$$

quantum number for Mn^{2+} ion ($\frac{5}{2}$), g and g_N are the g factors of the electron and proton, β and β_N are the Bohr magnetons of the electron and the proton, ω_I is the proton resonance frequency, ω_s is the electron resonance frequency, and τ_c is the dipolar correlation time. For the case under consideration, $\omega_s \gg \omega_I$ so that the second term in brackets can be neglected. Equation 2 then can be rearranged to give eq 3, where the known values of the natural constants have been inserted:

$$r = 815\left(\frac{T_{1M}3\tau_c}{1+\omega_I{}^2\tau_c{}^2}\right)^{1/6} \quad (3)$$

The appropriate value of $T_{1M}{}^{-1}$ is the difference between the values measured for the native and Mn-substituted enzymes, taking into account the fact that only half of the metal sites are occupied by Mn in the enzyme preparation used, i.e. twice the experimentally observed difference. In order to calculate the distance between manganese and the methylene protons of succinate with eq 3, the correlation time, τ_c, must be known. If τ_c is assumed to be frequency independent, it can be calculated from the observed frequency dependence of T_{1M} using eq 2. The value of τ_c obtained at 21° is 3.5×10^{-9} sec. If this value is substituted into eq 3, the distance between Mn and the succinate protons is found to be 15.3 Å.

The allosteric inhibitor CTP binds exclusively to the regulatory site of the enzyme in the presence of 2 mM carbamyl phosphate. In Figure 4, a plot of $(fT_{2p})^{-1}$ is plotted vs. temperature for the C-6 proton of CTP in the presence of native and Mn-substituted enzyme. The CTP concentration was 10^{-2} M and the enzyme concentration 3×10^{-5} M. The temperature dependence of the relaxation times suggests the fast exchange limit is valid, and within the experimental uncertainty the results obtained with native and Mn-substituted enzyme are the same. If the Mn is sufficiently close to CTP, the CTP resonance would be broadened by the Mn-substituted enzyme. In such a case the difference in the reciprocal transverse relaxation times can be described by eq 4 (Dwek, 1973). Since line broadening is not observed, it can be estimated that the Mn must be at least 20 Å from the CTP bound to the regulatory site. This estimate assumes the relaxation times of the diamagnetic and paramagnetic species must differ by at least 10% to be detected and that τ_c is equal to 3.5×10^{-9} sec.

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)g^2\beta^2g_N{}^2\beta_N{}^2}{r^6} \times \left[4\tau_c + \frac{3\tau_c}{1+\omega_I{}^2\tau_c{}^2} + \frac{13\tau_c}{1+\omega_s{}^2\tau_c{}^2}\right] \quad (4)$$

Discussion

The principal uncertainty in the calculated distance between manganese and bound ligands arises from the uncertainty in the correlation time. However, since the distance varies as the one-sixth power of the correlation time, fairly large changes in the correlation time would not markedly alter the calculated distance. Thus, the results reported here indicate that Mn is 15 Å from succinate protons and >20 Å from the C-6 proton of CTP bound to aspartate transcarbamylase.

The correlation time is determined by three relaxation times according to eq 5: the electron spin relaxation time,

$$\frac{1}{\tau_c} = \frac{1}{\tau_h} + \frac{1}{\tau_r} + \frac{1}{\tau_s} \quad (5)$$

τ_s, the rotational correlation time, τ_r, and the residence time of the bound ligand, τ_h. In the present case, τ_h is certainly greater than 10^{-6} sec. This can be estimated from the approximate equilibrium dissociation constant for succinate binding ($<10^{-3}$ M; Changeux and Rubin, 1968) and the maximum value of the second-order rate constant for association of ligand and enzyme ($\sim 10^9$ M^{-1} sec^{-1}), and also from directly measured kinetic constants (Sykes et al., 1970; Hammes and Wu, 1971). The correlation time obtained from the frequency dependence of T_{1M}, 3.5×10^{-9} sec, is considerably shorter than the rotational correlation time determined previously from nuclear magnetic resonance measurements, 2×10^{-8} sec (Sykes et al., 1970). This suggests that the correlation time may be mainly determined by the electron spin relaxation time. However, the temperature and frequency dependence of T_1 for succinate binding to the Zn-enzyme suggest the rotational correlation time of bound succinate is shorter than 2×10^{-8} sec. At this time, the reason for this discrepancy is not clear; however, only the difference in relaxation times between diamagnetic and paramagnetic species is of importance in this work.

The implicit assumption has been made that Mn binds to the enzyme specifically at the Zn binding site. The stoichiometry of the metal analyses strongly suggests Mn is substituting directly for Zn. Moreover, the purification procedure used for the Mn-enzyme would allow only very tightly bound metal ions to be retained, and the Zn binding site is the most likely site to bind Mn sufficiently tightly. Further information about the state of the bound Mn can be obtained from measurements of water relaxation times. Such experiments are somewhat tenuous because the instability of the Mn-substituted enzyme could produce some free Mn in solution. Preliminary experiments indicate that the transverse relaxation time of the HOD proton at 27° is shortened significantly in the presence of Mn-substituted enzyme. The relaxation time *increases* in the presence of carbamyl phosphate and succinate. Thus, the enzyme-bound Mn may be accessible to water, and if this is the case

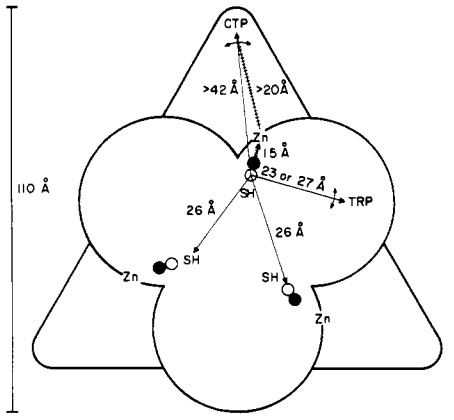

FIGURE 5: A schematic cross-sectional diagram of aspartate transcarbamylase showing the central catalytic trimer and the peripheral regulatory subunits. The single sulfhydryl group of the catalytic subunit is shown along with the proposed carbamyl phosphate (O), succinate (●), and CTP sites. The distances were determined from fluorescence energy transfer measurements (—, Matsumoto and Hammes, 1975) and the nuclear magnetic resonance measurements reported here (+++). The scale is only approximate.

the binding of carbamyl phosphate and succinate appears to cause a conformational change altering the environment of Mn.

The overall subunit structure of aspartate transcarbamylase can be depicted as follows (Evans et al., 1972, 1973, 1974; Warren et al., 1973; Richards and Williams, 1972). The enzyme is arranged as two trimeric catalytic subunits joined by three dimeric regulatory subunits such that each catalytic subunit is in contact with a regulatory subunit. The metal atom is located at the interface between a regulatory and catalytic subunit. Each catalytic subunit has a single sulfhydryl group, and X-ray studies indicate these sulfhydryl groups form an equilateral triangle (22 Å per side) on each catalytic trimer (the bottom and top of the native enzyme). Fluorescence energy transfer experiments (Matsumoto and Hammes, 1975) have indicated that an active site is adjacent to each sulfhydryl group and also have established the distances between active sites, between an active site and a nonadjacent sulfhydryl group, and between the active site and tryptophan on the catalytic subunit. A lower bound for the distance between the catalytic and regulatory sites also has been found. The results of the fluorescence energy transfer experiments and the distances determined in this work are summarized in Figure 5. In this figure, a schematic cross section of aspartate transcarbamylase is shown roughly based on the electron density map determined by X-ray diffraction (Warren et al., 1973). All of the results are self-consistent and consistent with the X-ray diffraction results. The X-ray studies indicate the sulfhydryl group is 24 Å from the Zn atom. All of the fluorescent analogs studied bind to the carbamyl phosphate site and are very close to the adjacent sulfhydryl group. In order for the distances to be self-consistent the sulfhydryl group is positioned at one end of the catalytic site, the carbamyl phosphate site then is placed adjacent to the sulfhydryl group between the sulfhydryl group and the Zn ion, and the succinate site is between the carbamyl phosphate and Zn atom. Molecular models indicate this would place succinate about midway between the sulfhydryl group and Zn atom, that is about 12 Å from each. The probes used are, of course, not point probes so that uncertainties of several angstroms in position are to be expected. Nevertheless, the use of fluorescence and paramagnetic probes has permitted the development of a quantitative structural map of aspartate transcarbamylase.

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References

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7,* 531.

Changeux, J.-P., and Rubin, M. M. (1968), *Biochemistry 7,* 553.

Cohlberg, J. A., Pigiet, V. P., and Schachman, H. K. (1972), *Biochemistry 11,* 3396.

Cook, R. A. (1972), *Biochemistry 11,* 3792.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry, Oxford, Clarendon Press, pp 174–242.

Evans, D. R., McMurray, C. H., and Lipscomb, W. N. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3638.

Evans, D. R., Pastra-Landis, S. C., and Lipscomb, W. N. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1351.

Evans, D. R., Warren, S. G., Edwards, B. F. P., McMurray, C. H., Bethge, P. H., Wiley, D. C., and Lipscomb, W. N. (1973), *Science 179,* 683.

Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242,* 2886.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237,* 891.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4,* 1054.

Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7,* 538.

Gray, C. W., Chamberlin, M. J., and Gray, D. M. (1973), *J. Biol. Chem. 248,* 6071.

Hammes, G. G., Porter, R. W., and Wu, C.-W. (1970), *Biochemistry 9,* 2292.

Hammes, G. G., and Wu, C.-W. (1971), *Biochemistry 10,* 1051.

Hammes, G. G., and Wu, C.-W. (1974), *Annu. Rev. Biophys. Bioeng. 3,* 1.

Jacobson, G. R., and Stark, G. R. (1973), *Enzymes, 3rd Ed., 9,* 225.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40,* 2686.

Matsumoto, S., and Hammes, G. G. (1973), *Biochemistry 12,* 1388.

Matsumoto, S., and Hammes, G. G. (1975), *Biochemistry 14,* 214.

McDonald, G. G., and Leigh, J. S. (1973), *J. Magn. Reson. 9,* 358.

Mildvan, A. S., and Cohn, M. (1970), *Adv. Enzymol. Relat. Areas Mol. Biol. 33,* 1.

Nelbach, M. E., Pigiet, V. P., Jr., Gerhart, J. C., and Schachman, H. K. (1972), *Biochemistry 11,* 315.

Prescott, L. M., and Jones, M. E. (1969), *Anal. Biochem. 32,* 408.

Richards, K. E., and Williams, R. C. (1972), *Biochemistry*

11, 3393.
Rosenbusch, J. P., and Griffin, J. H. (1973), *J. Biol. Chem. 248,* 5063.
Rosenbusch, J. P., and Weber, K. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1019.
Schachman, H. K. (1972), in Protein-Protein Interactions, Jaenicke, R., and Helmreich, E., Ed., New York, N.Y., Springer-Verlag New York.
Solomon, I. (1955), *Phys. Rev. 99,* 559.
Suzuki, M., and Wacker, W. F. C. (1974), *Anal. Biochem. 57,* 605.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37,* 307.
Sykes, B. D., Schmidt, P. G., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 1180.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5,* 273.
Warren, S. G., Edwards, B. F. P., Evans, D. R., Wiley, D. C., and Lipscomb, W. N. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1117.
Weber, K. (1968), *Nature (London) 218,* 1116.
Winlund, C. C., and Chamberlin, J. J. (1970), *Biochem. Biophys. Res. Commun. 40,* 43.
Wu, C.-W., and Hammes, G. G. (1973), *Biochemistry 12,* 1400.

Kinetics of Glycogen Phosphorylase *a* with a Series of Semisynthetic, Branched Saccharides. A Model for Binding of Polysaccharide Substrates†

Hsiang-Yun Hu and Allen M. Gold*

ABSTRACT: The requirement of muscle phosphorylase for branched polysaccharide substrates was investigated by kinetic studies on semisynthetic branched saccharides. One series of saccharides was prepared from maltoheptaose by oxidizing the reducing group to a carboxyl group and coupling this with an amino group of ethylenediamine. The resulting aminooligosaccharide was coupled with *p*-nitrophenyl esters of mono-, di-, tetra-, and polycarboxylic acids to produce saccharides containing one, two, four, and approximately 52 maltodextrin chains per molecule. A similar series of saccharides was prepared from a heterogeneous maltodextrin of average chain length 11.7. Kinetic constants were determined for the reaction with phoshorylase *a* in the direction of chain elongation. Michaelis constants are equilibrium constants for dissociation of saccharide from the enzyme-AMP-glucose-1-P-saccharide complex. The Michaelis constants, expressed in terms of the concentration of nonreducing end groups, are independent of maltodextrin chain length but decrease considerably as the number of chains per molecule increases. Maximum velocities do not differ greatly from that for glycogen. Among the synthetic saccharides, only the polymer behaves similarly to glycogen in exhibiting a decreasing reaction rate as the chains are elongated. The kinetic constants are quantitatively consistent with a model in which two chain termini from the same saccharide molecule bind to the phosphorylase molecule simultaneously. Differences in binding between saccharides having different numbers of equally accessible chains are caused solely by statistical factors in the equilibrium. Highly branched substrates bind better because of their greater multiplicity of two end-group pairs.

Glycogen phosphorylase (EC 2.4.1.1, α-1,4-glucan:orthophosphate glucosyltransferase) from rabbit skeletal muscle catalyzes the reversible transfer of an α-glucosyl group, with retention of configuration, between orthophosphate and the 4 position of the nonreducing terminus of an α-1,4-glucan. The enzyme shows considerable specificity for highly branched α-1,4-glucans such as glycogen and amylopectin, which contain α-1,6 branch points, compared to linear glucans like simple maltodextrins and amylose (Goldemberg, 1962; Smith, 1971). Specificity is manifested largely in the Michaelis constants for the glucans; the branched polysaccharides have far smaller K_m values than do maltodextrins, such as maltotetraose or maltopentaose. Maximum velocities are comparable for the two types of saccharide. The kinetic mechanism of phosphorylases *a* and *b* is known to be rapid equilibrium random bi-bi (Gold et al., 1970; Engers et al., 1969, 1970a,b) and the Michaelis constant is the equilibrium constant for dissociation of the saccharide from a central complex.

In contrast, potato phosphorylase, which has physical and kinetic properties very similar to those of muscle phosphorylase (Gold et al., 1971), shows little or no discrimination between branched and linear saccharides. When the natural substrate, amylopectin, is debranched enzymatically the resulting mixture of maltodextrins is as good a substrate as the original amylopectin (Smith, 1971).

In the present work we have attempted to answer the question of why muscle phosphorylase shows such great preference for binding branched α-1,4-glucans by studying the kinetics of phosphorylase *a* with a series of semisynthet-

† From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received December 4, 1974.* Taken in part from a dissertation submitted by H.-Y.H. to the Graduate School of Arts and Sciences of Columbia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This work was supported by a research grant from the National Science Foundation (GB-31289). H.-Y.H. was supported by a Graduate Fellowship from Columbia University. A preliminary report on this work appeared in *Fed. Proc. Fed. Am. Soc. Exp. Biol. 33,* 1375 (1974).