Reciprocal Cooperative Effects of Multiple Ligand Binding to Pyruvate Kinase†

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ABSTRACT: The formation of multiple ligand complexes with muscle pyruvate kinase was measured in terms of dissociation constants and the standard free energies of formation were calculated. The binding of Mn^{2+} to the enzyme ($K_A = 55 \pm 5 \times 10^{-6}$ M; $\Delta F° = -5.75 \pm 0.05$ kcal/mol) and to the enzyme saturated with phosphoenolpyruvate (conditional free energy) ($K_A' = 0.8 \pm 0.4 \times 10^{-6}$ M; $\Delta F° = -8.22 \pm 0.34$ kcal/mol) has been measured under identical conditions giving a free energy of coupling, $\Delta(\Delta F°) = -2.47 \pm 0.34$ kcal/mol. Such a large negative free energy of coupling is diagnostic of a strong positively cooperative effect in ligand binding. The binding of the substrate phosphoenolpyruvate to free enzyme and the enzyme-Mn^{2+} complex was, by necessity, measured by different methods. The free energy of phosphoenolpyruvate binding to free enzyme ($K_S = 1.58 \pm 0.10 \times 10^{-4}$ M; $\Delta F° = -5.13 \pm 0.04$ kcal/mol) and to the enzyme-Mn^{2+} complex ($K_3 = 0.75 \pm 0.10 \times 10^{-6}$ M; $\Delta F° = -8.26 \pm 0.07$ kcal/mol) also gives a large negative free energy of coupling, $\Delta(\Delta F°) = -3.16 \pm 0.08$ kcal/mol. Such a large negative value confirms reciprocal binding effects between the divalent cation and the substrate phosphoenolpyruvate. The binding of Mn^{2+} to the enzyme-ADP complex was also investigated and a free energy of coupling, $\Delta(\Delta F°) = -0.08 \pm 0.08$ kcal/mol, was measured, indicative of little or no cooperativity in binding. The free energy of coupling with Mn^{2+} and pyruvate was measured as -1.52 ± 0.14 kcal/mol, showing a significant amount of cooperativity in ligand binding but a substantially smaller effect than that observed for phosphoenolpyruvate binding. The magnitude of the coupling free energy may be related to the role of the divalent cation in the formation of the enzyme-substrate complexes. In the absence of the activating monovalent cation, the coupling free energies for phosphoenolpyruvate and pyruvate binding decrease by 40-60% and 25%, respectively, substantiating a role for the monovalent cation in the formation of enzyme-substrate complexes with phosphoenolpyruvate and with pyruvate.

The phenomenon of cooperativity in ligand binding to a protein by the binding of a second ligand has been well documented. Since the first observations made with binding to hemoglobin (Antonini et al., 1963), these reciprocal effects of mutual ligand binding have also been observed for a large number of enzymes. These effects have been treated in a qualitative fashion to describe the mutual effects occurring in multiligand binding. Recently, a quantitative thermodynamic expression for mutual ligand effects on ligand binding has been derived (Weber, 1972) and applied to the binding of oxalate and NADH[1] to lactate dehydrogenase (Kolb and Weber, 1975). Their measurements for multiple ligand binding demonstrated cooperativity in binding by both ligands to the enzyme.

A substantial amount of information has been obtained concerning the formation and structures of enzyme-metal-ligand complexes with pyruvate kinase (for review, see Kayne, 1973). Kinetic studies have indicated an independence of interaction of phosphoenolpyruvate and of ADP to the enzyme (Reynard et al., 1961); however, a cooperative effect between Mn^{2+} and ADP and Mn^{2+} and phosphoenolpyruvate was indicated (Mildvan and Cohn, 1966). Kinetic studies have also indicated a mutually cooperative effect between binding of the activating divalent cation and the activating monovalent cation (Suelter et al., 1966; Nowak, 1973). Binding studies have also shown these reciprocal effects to occur with the binding of phosphoenolpyruvate and of K^+ to the enzyme-Mn^{2+} complex (Nowak and Mildvan, 1972). Thus, with pyruvate kinase the cooperative interactions which occur with ligand binding appear to be primarily modulated by the cations required for activity, and the ligand most strongly affected is phosphoenolpyruvate.

In the present investigation, we have attempted to measure the effects of substrate ligands on metal binding, and vice versa, where feasible, by direct binding studies. We have demonstrated that a strong cooperative effect exists between the binding of phosphoenolpyruvate and Mn^{2+} to pyruvate kinase. However, a negligible effect of ADP on Mn^{2+} binding exists. The binding of pyruvate and Mn^{2+} occur in a mutually cooperative manner but not as strong as with phosphoenolpyruvate and Mn^{2+} binding. The presence of K^+ increases the magnitude of the cooperative interaction in the formation of both the ternary enzyme-Mn^{2+}-phosphoenolpyruvate and enzyme-Mn^{2+}-pyruvate complexes.

Materials and Methods

Rabbit muscle pyruvate kinase and lactate dehydrogenase were purchased from Boehringer. The TMA-Cl and TMA-OH were obtained from Eastman and recrystallized twice before use. The phosphoenolpyruvate, ADP, pyruvate, pyruvic acid, and NADH were purchased from Sigma.

Pyruvate kinase was assayed by the method of Teitz and Ochoa (1958) as previously modified (Nowak and Mildvan, 1972). Prior to use, the enzyme was desalted by passage through a Sephadex G-25 fine column and, when necessary, concentrated using a collodion bag, vacuum filtration appa-

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[1] Abbreviations used are: TMA, tetramethylammonium cation; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; PRR, water proton nuclear longitudinal relaxation rate; NADH, nicotinamide adenine dinucleotide; ADP, adenosine diphosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

ratus. The enzyme concentration was determined using an extinction coefficient $E_{280}^{1\%} = 5.4\ cm^{-1}$ and a molecular weight of 237 000 (Warner, 1958). The binding of phosphoenolpyruvate was performed by the technique of Hummel and Dreyer (1962). A Sephadex G-25 fine column was prepared in a disposable pipet (5 × 70 mm) and equilibrated with buffer containing 50 mM Tris-Cl, 0.1 M KCl, and an appropriate concentration of phosphoenolpyruvate at pH 7.5. The enzyme was added to the column in a 0.1-mL volume and eluted with the equilibrating buffer. Fractions were collected at every two drops. The volume of the fractions was calibrated by preweighing the collecting tubes and then reweighing the filled tubes. Each fraction usually contained 66 ± 2 μL volume. The concentration of phosphoenolpyruvate in each tube was measured enzymatically via a modification of the assay procedure, making phosphoenolpyruvate the limiting substrate. The area under the elution peak was integrated in terms of the total micromoles of phosphoenolpyruvate bound in each experiment. The area in the peak (in terms of micromoles of phosphoenolpyruvate) was compared to the area measured in the trough, and any discrepancy was usually less than 5%. The data were calculated in terms of concentration of phosphoenolpyruvate bound per concentration of enzyme in the peak.

The binding of Mn^{2+} to pyruvate kinase and pyruvate kinase-ligand complexes was performed using PRR and EPR measurements. The $1/T_1$ measurements were obtained with a Seimco pulsed NMR spectrometer operating at 24.3 MHz at room temperature (22 ± 1 °C) using the Carr-Purcell 180°-τ-90° sequence (1954). Individual sample tubes were prepared containing a fixed concentration of enzyme in Tris buffer, pH 7.5, containing either 0.1 M KCl or 0.1 M TMA-Cl where noted, a fixed amount of substrate where indicated, and varying concentrations of Mn^{2+} in a total volume of 0.05 mL. A series of control tubes prepared in the absence of enzyme (and ligand) was also used and, from the measured relaxivity, the absolute total concentration of Mn^{2+} is measured in each tube. The effect of the bound Mn^{2+} on the enhancement (ϵ) of the $1/T_1$ relaxation rate of water protons is calculated as the ratio of the paramagnetic effect of Mn^{2+} ($1/T_{1p} = 1/T_{1,obsd} - 1/T_{1,0}$) in the presence and in the absence of a macromolecule.

$$\epsilon^* = \frac{1/T_{1p}^*}{1/T_{1p}} = \frac{(1/T_{1,obsd}^* - 1/T_{1,0}^*)}{(1/T_{1,obsd} - 1/T_{1,0})} \quad (1)$$

The presence of a macromolecule is designated with as asterisk. This parameter has been previously defined (Mildvan and Cohn, 1970). The designated ϵ^* refers to the observed enhancement for each particular experiment. After PRR measurements, the samples were placed in a quartz capillary tube (1-mm i.d.) and the EPR spectra were recorded on a Varian V-4500 X-band EPR spectrometer recording the spectra at a 1 kG range. The probe temperature was kept constant with N_2 flow at 22 ± 1 °C. The signal amplitude (the sum of the six line heights), normalized for the gain level, was plotted as a function of Mn^{2+} concentration for the control samples. The straight lines obtained served as a standard curve. The spectrum amplitude measured in the presence of enzyme (or ligand) served as a measure of free Mn^{2+}. The limits of our sensitivity were between 5–10 × 10^{-6} M free Mn^{2+}. Studies performed with enzyme-ligand complexes were usually done at higher enzyme concentrations to increase the concentration of the enzyme-ligand binary complex and minimize the effect of Mn^{2+} binding by free ligand. These effects were more striking with ligands which have a K_d for Mn^{2+} comparable to that of the enzyme (i.e., ADP and ATP). Such experiments were usually performed at several concentrations of enzyme and several concentrations of ligand. From measurements of $[Mn^{2+}]_f$ and observed enhancement values ϵ^* measured by PRR, the binary ϵ_b, ternary ϵ_T, and quaternary ϵ_q enhancements can be estimated. Such estimates are based on the relationship for the binary complex:

$$\epsilon^* = \left(\frac{[Mn^{2+}]_f}{[Mn^{2+}]_T}\right)\epsilon_a + \left(\frac{[Mn^{2+}]_b}{[Mn^{2+}]_T}\right)\epsilon_b \quad (2)$$

where ϵ^* is the observed enhancement, ϵ_a is the enhancement of free Mn^{2+}, defined as 1, and ϵ_b is the binary enhancement. In the case of formation of ternary complexes, an analogous relationship holds:

$$\epsilon^* = \frac{[Mn^{2+}]_f}{[Mn^{2+}]_T} + \left(\frac{[Mn^{2+}]_b}{[Mn^{2+}]_T}\right)\epsilon_b + \left(\frac{[Mn^{2+}]_c}{[Mn^{2+}]_T}\right)\epsilon_T \quad (3)$$

where ϵ_T is the enhancement of the ternary complex and $[Mn^{2+}]_c$ is the concentration of Mn^{2+} in the ternary complex. Under conditions where saturation is obtained, $[Mn^{2+}]_b$ approaches zero; thus, ϵ_T can be easily estimated directly from the PRR and EPR data.

The binding studies were interpreted in terms of the Scatchard equation (1949):

$$[Mn^{2+}]_b/[E]_T[Mn^{2+}]_f = \frac{n}{K_d} - [Mn^{2+}]_b/[E]_T K_d \quad (4)$$

where the subscripts b, f, and T represent bound, free, and total manganese or enzyme, respectively, K_d is the dissociation constant, and n is the number of equivalent binding sites. The data is reported along with the standard deviation calculated from data obtained from several such experiments.

The PRR titration studies were performed as previously described (Nowak and Mildvan, 1970) and the dissociation constant for phosphoenolpyruvate binding to the E-Mn^{2+} complex was evaluated by fitting the data via computer analysis (Reed et al., 1970) using the parameters independently measured in this study. Such analyses of PRR binding studies have been previously described (Nowak and Mildvan, 1972).

Thermodynamic Treatment of the Data. The thermodynamic relationships used to express ligand and metal binding were based on the general relationships derived for ligand binding to proteins by Weber (1972). The apparent free-energy change for binding of metal ($\Delta F°_M$) or binding of ligand ($\Delta F°_L$) to form binary enzyme-metal or enzyme-ligand complexes can usually be measured. When metal binding to the enzyme-ligand complex or ligand binding to the enzyme-metal complex can be measured, their respective conditional free energies for binding ($\Delta F°_{M/L}$ and $\Delta F°_{L/M}$) can also be estimated. From the principle of conservation of free energy, assuming the formation of the ternary enzyme-metal-ligand is a random process and the E-M-L complex is independent of the path of formation, then

$$\Delta F°_{M/L} - \Delta F°_M = \Delta F°_{L/M} - \Delta F°_L \quad (5)$$

This relationship can also be described by considering the four relationships

$$K_A = \frac{[E][M]}{[EM]} \quad (6)$$

$$K_S = \frac{[E][L]}{[EL]} \quad (7)$$

$$K_A' = \frac{[EL][M]}{[EML]} \quad (8)$$

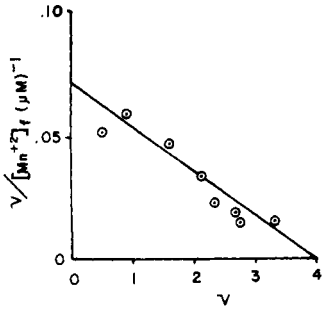

FIGURE 1: Scatchard plot of the binding of Mn^{2+} to pyruvate kinase. The concentration of enzyme used was 4.18×10^{-5} M and the concentrations of $MnCl_2$ used varied from 3×10^{-5} M to 3.5×10^{-4} M. $T = 22 \pm 1$ °C. $\nu = [Mn^{2+}]_b/[E]_T$.

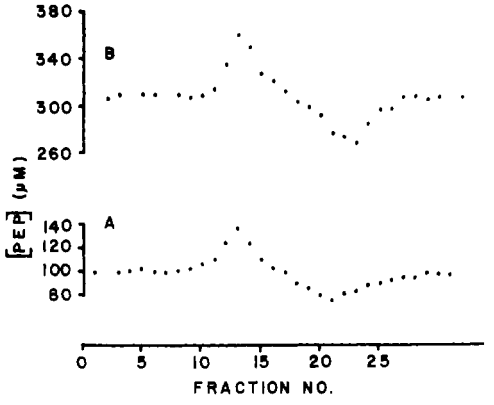

FIGURE 2: Elution profiles of enzyme-phosphoenolpyruvate binding studies. A 0.10-mL sample of pyruvate kinase (7.5×10^{-5} M) was placed on a Sephadex G-25 fine column previously equilibrated with phosphoenolpyruvate at 1.0×10^{-4} M (A) or 3.1×10^{-4} M (B). Fractions (0.065 mL) were collected and assayed for phosphoenolpyruvate. $T = 22 \pm 1$ °C.

$$K_3 = \frac{[EM][L]}{[EML]} \tag{9}$$

From these equations it can be shown that

$$K_A K_3 = K_S K_A' \tag{10}$$

Considering we can calculate $\Delta F°_M$ from K_A, $\Delta F°_L$ from K_S, $\Delta F°_{M/L}$ from K_A', and $\Delta F°_{L/M}$ from K_3, we arrive at the same relationships. Under conditions of independent binding of M and L, $\Delta(\Delta F°) = \Delta F°_{M/L} - \Delta F°_M = 0$; for positively cooperative binding $\Delta(\Delta F°) < 0$ and for negatively cooperative binding $\Delta(\Delta F°) > 0$. This difference in free energies is a quantitative measure of the degree of mutual interdependence of binding and a thermodynamic measure of cooperativity. This coupling free energy, or free energy of cooperativity, can thus be used to demonstrate the degree of cooperativity occurring in the formation of enzyme-metal-ligand complexes.

Results

Binary Enzyme-Ligand Complexes. The binding of Mn^{2+} to pyruvate kinase was measured using PRR and EPR techniques and the data are presented in the form of a Scatchard plot (Figure 1). Our studies show four independent binding sites for Mn^{2+} per mole of enzyme with a dissociation constant $K_A = 55 \pm 5 \times 10^{-6}$ M and a binary enhancement $\epsilon_b = 25.0 \pm 1.3$. These values are in excellent agreement with those values reported earlier (Reuben and Cohn, 1970; Cottam and Mildvan, 1971) and substantiate the presence of four independent divalent cation sites per enzyme.

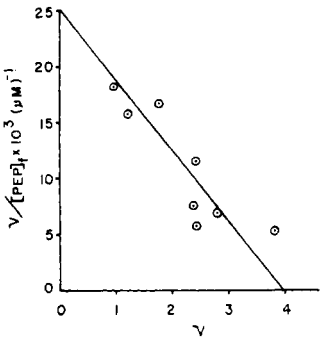

FIGURE 3: Scatchard plot of the binding of phosphoenolpyruvate to pyruvate kinase. The concentration of enzyme used was 7.5×10^{-5} M and the concentrations of phosphoenolpyruvate used varied from 4.9×10^{-5} to 6.8×10^{-4} M. The concentration of phosphoenolpyruvate bound was obtained from integration of the peak as shown in Figure 2. $\nu = [phosphoenolpyruvate]_b/[E]_T$. $T = 22 \pm 1$ °C.

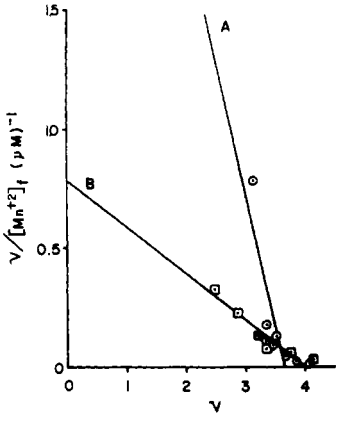

FIGURE 4: Scatchard plot of the binding of Mn^{2+} to the pyruvate kinase-phosphoenolpyruvate complex. The experiments were done with 4.5×10^{-5} M enzyme, varying the total amount of $MnCl_2$ from 1.19×10^{-4} to 3.98×10^{-4} M in (A) the presence of 0.1 M KCl (⊙) or (B) of 0.1 M TMA-Cl (⊡). $T = 22 \pm 1$ °C. $\nu = [Mn^{2+}]_b/[E]_T$.

The binding of substrate phosphoenolpyruvate to pyruvate kinase was also studied to obtain a direct measure of a value for the K_S of the substrate. It had previously been reported that four substrate sites also exist on pyruvate kinase (Kayne, 1971), although an earlier report indicated two sites with $K_S = 2.5 \times 10^{-5}$ M (Reynard et al., 1961). Initial attempts at binding studies using equilibrium dialysis proved to be unreliable and nonreproducible in our hands and thus we used the technique of Hummel and Dreyer (1962). This technique gave us results which were quite reproducible and were rather sensitive. Elution profiles for several experiments at various phosphoenolpyruvate concentrations are shown in Figure 2. After integrating the curves for each experiment, the results are presented in the form of a Scatchard Plot (Figure 3). A value of 3.9 ± 0.1 substrate sites was obtained, in contrast to 2.2 sites measured earlier by equilibrium dialysis (Reynard et al., 1961) but consistent with the value of 3.8 ± 0.3 sites per mole also measured by equilibrium dialysis (Kayne, 1971). The dissociation constant, K_S, was estimated to be $1.58 \pm 0.10 \times 10^{-4}$ M.

The Binding of Mn^{2+} to Binary Enzyme-Substrate Complexes. Kinetic studies have indicated a mutual interrelationship in interaction between the substrate phosphoenolpyruvate and Mn^{2+} with pyruvate kinase (Mildvan and Cohn,

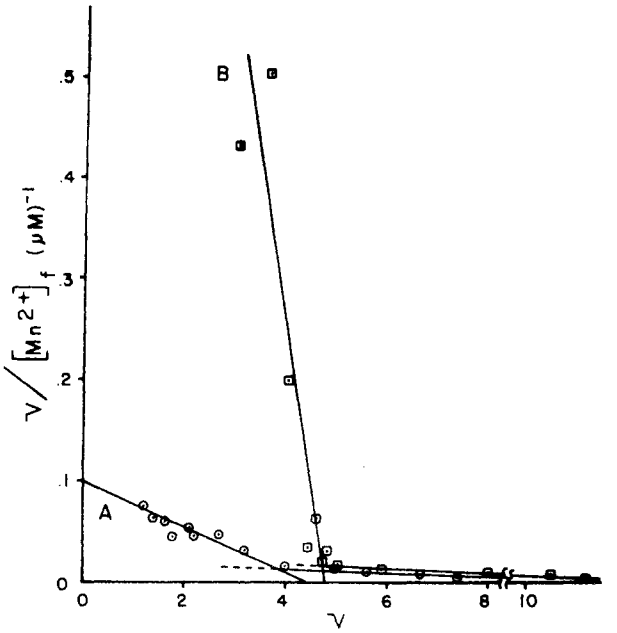

FIGURE 5: Scatchard plots of Mn^{2+} binding to the pyruvate kinase–ADP binary complex and the pyruvate kinase–ATP–pyruvate ternary complex. The conditions used to study the binary complex were 9.1×10^{-5} M enzyme in the presence of 7.5×10^{-4} M ADP, varying Mn^{2+} from 1.25×10^{-4} to 2×10^{-3} M (⊙) (A). To study the quaternary complex, 9.84×10^{-5} M enzyme was used in the presence of 10^{-2} M pyruvate and 4×10^{-4} M ATP with Mn^{2+} varying from 3×10^{-4} to 5×10^{-3} M (⊡) (B). $T = 22 \pm 1$ °C. $\nu = [Mn^{2+}]_b/[E]_T$.

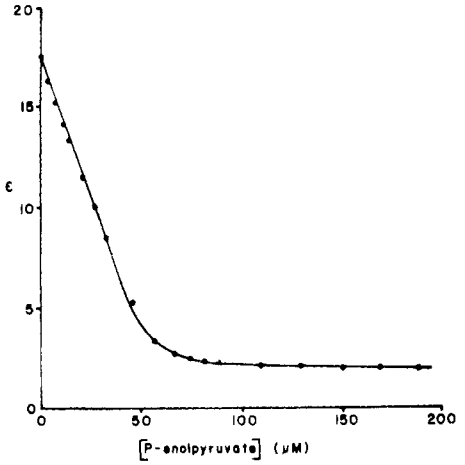

FIGURE 6: Titration of pyruvate kinase–Mn^{2+} with phosphoenolpyruvate. A plot of observed enhancement (ϵ^*) vs. phosphoenolpyruvate concentration is made. A solution containing 4.58×10^{-5} M enzyme in 50 mM Tris buffer, pH 7.5, containing 0.1 M KCl and 5×10^{-5} M Mn^{2+} is titrated with a solution containing an identical solution and including 1.98×10^{-4} M phosphoenolpyruvate. The PRR of water was made after each addition and the enhancement values calculated as described. $T = 22 \pm 1$ °C.

1966). To demonstrate this interrelationship directly and to demonstrate the reciprocal relationships with should occur in ligand binding (Kolb and Weber, 1975), the formation of these higher complexes was investigated. Pyruvate kinase, under conditions of saturation with phosphoenolpyruvate, was found to bind Mn^{2+} more tightly than free enzyme (Figure 4A). Conditions were controlled such that the enzyme was fully saturated and a minimal amount of Mn^{2+} binding by free phosphoenolpyruvate occurred. A more extensive binding curve for this complex is limited by the sensitivity of the EPR spectrometer to detect free Mn^{2+}; however, results from eight

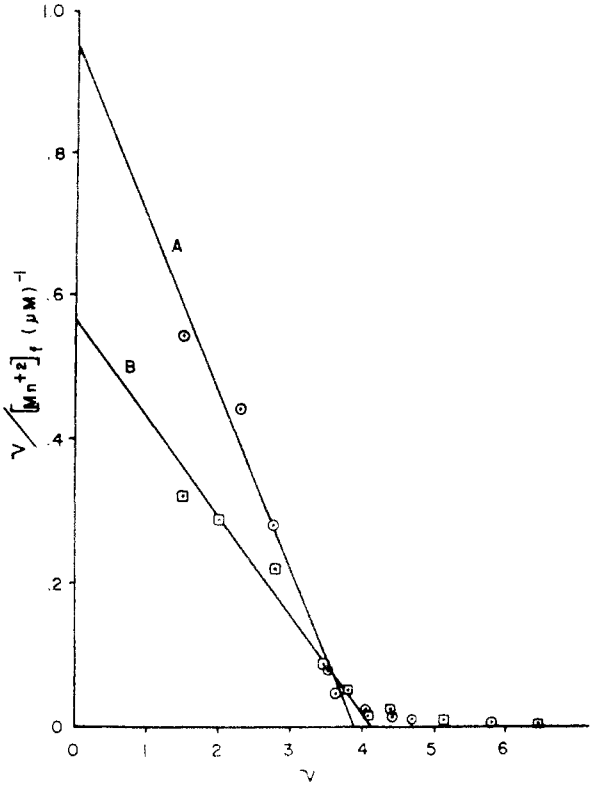

FIGURE 7: Scatchard plots of Mn^{2+} binding to the pyruvate kinase–pyruvate complex in the presence and absence of KCl. The experiments were performed using 3×10^{-5} M enzyme and 10 mM pyruvate in the presence of 0.1 M KCl (⊙) or 0.1 M TMA-Cl (⊡) $T = 22 \pm 1$ °C and $\nu = [Mn^{2+}]_b/[E]_T$.

different experiments give values of $n = 3.7 \pm 0.3$ sites and $K_A' = 0.8 \pm 0.4 \times 10^{-6}$ M. Analogous experiments were performed with the pyruvate kinase–ADP complex. These studies were complicated by the fact that the enzyme has a higher K_S for ADP than for phosphoenolpyruvate (Mildvan and Cohn, 1966), making a greater concentration of ADP necessary for saturation to be obtained. However, since ADP has a greater affinity for Mn^{2+} than does phosphoenolpyruvate (K_d ADP $= 115 \pm 10$ μM, $n = 1.05$ vs. K_d phosphoenolpyruvate $= 1.9 \pm 0.2 \times 10^{-3}$ M, $n = 1.02$ measured under our conditions), binding of Mn^{2+} by free ADP becomes a factor. This complication has been minimized by increasing the concentration of enzyme used at a fixed ADP concentration. Results of one such binding experiment are shown in Figure 5A. The value of $K_A'' = 45 \times 10^{-6}$ M and $n_1 = 4.5$. A second set of sites is observed; in this experiment, $n_2 = 4.9$ and $K_{dissoc} = 3.5 \times 10^{-4}$ M. At the same concentration of enzyme and increasing ADP, n_1 increases and K_A'' decreases, an artifact of additional competition of Mn^{2+} binding by ADP. Under these same conditions, the value of n_2 also increases with a concomitant decrease in K_{dissoc}, also an artifact of high ADP concentration. As a limiting value at high enzyme concentrations with >80% saturation, $n \approx 4$ and $K_A'' = 48 \pm 5 \times 10^{-6}$ M and $\epsilon_t = 17.5 \pm 1.5$. The formation of a pyruvate kinase–ADP complex does not cause a significant effect in either n or K_A.

Since the formation of such complexes with E–ATP would be even further complicated because ATP has a greater affinity for Mn^{2+} ($K_{dissoc} = 18 \pm 3 \times 10^{-6}$ M, $n = 0.95$), the investigation of such complexes was not rigorously pursued. Several initial experiments at 32 μM enzyme and 500 μM ATP gave values of $n = 15$ and $K_d = 25$ μM.

Formation of the Ternary Pyruvate Kinase–Mn^{2+}–Phos-

TABLE I: Binding Constants for Pyruvate Kinase Complex Formation.[a]

Enzyme Complex Titrated	Titrant	n (mol bound/ mol of enzyme)	$K_{dissoc} \times 10^6$ (M)	$\Delta F°$ (kcal/mol)
PK[b]	Mn^{2+}	3.9 ± 0.1	55 ± 5	−5.75 ± 0.05
PK[c]	Mn^{2+}	4.1 ± 0.1	49 ± 5	−5.82 ± 0.06
PK[b]	P-enolpyruvate	3.9 ± 0.1	158 ± 10	−5.13 ± 0.04
PK-P-enolpyruvate[b]	Mn^{2+}	3.7 ± 0.3	0.8 ± 0.4	−8.23 ± 0.34
PK-P-enolpyruvate[c]	Mn^{2+}	4.0 ± 0.1	5.0 ± 1.0	−7.15 ± 0.10
PK-ADP[b]	Mn^{2+}	4	48 ± 5	−5.83 ± 0.06
PK-Mn^{2+} [b]	P-enolpyruvate		0.75 ± 0.10	−8.26 ± 0.07
PK-Mn^{2+} [c,d]	P-enolpyruvate		3.9 ± 0.4	−7.30 ± 0.06
PK-Mn^{2+} [b,d]	pyruvate		190 ± 40	−5.02 ± 0.11
PK-Mn^{2+} [c,d]	pyruvate		660 ± 170	−4.29 ± 0.13
PK-pyruvate[b]	Mn^{2+}	3.9 ± 0.1	4.1 ± 1.0	−7.27 ± 0.13
PK-pyruvate[c]	Mn^{2+}	4.1 ± 0.1	7.3 ± 0.7	−6.93 ± 0.05
PK-P-enolpyruvate-ADP[b]	Mn^{2+}	4.6 ± 0.3	2.6 ± 0.5	−7.54 ± 0.11

[a] These parameters were measured under conditions kept as identical as possible. Abbreviations used: PK, pyruvate kinase; P-enolpyruvate, phosphoenolpyruvate. [b] Measured in the presence of 0.1 M KCl. [c] Measured in the presence of 0.1 M TMA-Cl. [d] Data taken from Nowak and Mildvan (1972).

phoenolpyruvate Complex. In order to compare the dissociation constants for the formation of the various interacting ligand complexes, the binding of phosphoenolpyruvate to the enzyme-Mn^{2+} complex (K_3) was repeated under our experimental conditions (Figure 6) by PRR titration. The data were fit by an iterative process using the parameters independently measured and reported in this work, and a value of $K_3 = 0.75 \pm 0.10 \times 10^{-6}$ M was calculated, in agreement with previously reported values for K_3 (Nowak and Mildvan, 1972; James et al., 1973).

The Formation of Enzyme-Mn^{2+}-Pyruvate Ternary Complexes. Although kinetic studies analogous to those performed with phosphoenolpyruvate and ADP as substrates have not been performed using pyruvate and ATP because of the unfavorable $\Delta F°'$ for the net reaction, pyruvate has been shown to interact with the enzyme and the enzyme-Mn^{2+} complex (Mildvan and Cohn, 1966; Nowak and Mildvan, 1972; Fung et al., 1973; Reed and Cohn, 1973; James and Cohn, 1974). Although pyruvate binds more than two orders of magnitude more weakly to the enzyme-Mn^{2+} complex (Nowak and Mildvan, 1972), its effect on the structure of Mn^{2+} in the ternary complex is analogous to the effect caused by phosphoenolpyruvate binding (Reed and Cohn, 1973). The effect of the pyruvate kinase-pyruvate complex on the binding of Mn^{2+} is shown in Figure 7A. The enzyme-pyruvate complex binds Mn^{2+} with a $K_d = 4.1 \pm 1 \times 10^{-6}$ M, $n = 3.9 \pm 0.1$, and $\epsilon_t = 5.6 \pm 0.5$, an increase in Mn^{2+} binding to pyruvate kinase by an order of magnitude.

The Binding of Mn^{2+} to the Quaternary Enzyme Complex. It has been observed that the structure of Mn^{2+} in the enzyme-Mn^{2+}-pyruvate complex is quite similar, if not identical, to its structure in the equilibrium complex, as judged by EPR studies (Reed and Cohn, 1973), although the structures of the active sites of these complexes are significantly different (T. Nowak, unpublished observations). The binding of Mn^{2+} to the equilibrium complex was measured (Figure 5B) and shown to have 4.6 ± 0.3 Mn^{2+} sites per mole with $K_{dissoc} = 2.6 \pm 0.5 \times 10^{-6}$ M $\epsilon_q = 6.0 \pm 0.5$. Under catalytic conditions, the observed weak sites, analogous to those seen with the enzyme-ADP complex, are insignificant; the best fit to the weaker sites observed gives $n_2 = 21.5$ and $K_d' = 1.4 \times 10^{-3}$ M. Thus, under catalytically active conditions the enzyme-substrates complex binds Mn^{2+} as tightly as the enzyme-pyruvate complex but weaker than the enzyme-phosphoenolpyruvate complex.

The Effect of K^+ on Mn^{2+} Binding. It has been observed that the presence of K^+ increases the affinity of the enzyme-Mn^{2+} complex for several ligands, including phosphoenolpyruvate and pyruvate (Nowak and Mildvan, 1972). The monovalent cation also affects the structure of Mn^{2+} in the enzyme-Mn^{2+}-phosphoenolpyruvate complex, however, not in the enzyme-Mn^{2+}-pyruvate complex (Reed and Cohn, 1973). The effect of K^+ on Mn^{2+} binding was thus also studied. It has previously been reported (Reuben and Cohn, 1970; Nowak and Mildvan, 1972) that the presence or absence of an activating monovalent cation has no effect on the binding of Mn^{2+} to pyruvate kinase. This observation was again substantiated (with K^+: $n = 3.9 \pm 0.1$, $K_A = 55 \pm 5 \times 10^{-6}$ M, $\epsilon_b = 25.0 \pm 1.3$; with TMA^+: $n = 4.1 \pm 0.1$, $K_A = 49 \pm 5 \times 10^{-6}$ M, $\epsilon_b = 24.4 \pm 1.4$).

In the study of the enzyme-phosphoenolpyruvate complex, it was observed (Figure 4B) that, in the absence of K^+, Mn^{2+} bound less tightly ($n = 4.0$, $K_d = 5 \pm 1 \times 10^{-6}$ M) than in the presence of K^+ by a factor of 5. The ternary enhancement also increased from about 2 to 7 as previously observed (Nowak and Mildvan, 1972). This change in binding was also observed with pyruvate as the ligand but with a less dramatic effect (Figure 7B). The absence of K^+ decreases the affinity of the enzyme-pyruvate to complex Mn^{2+} by a factor of 2 ($K_d = 7.3 \pm 0.7 \times 10^{-6}$ M, $n = 4.1 \pm 0.1$). Thus, the presence of an activating monovalent cation affects the binding of certain ligands to the enzyme-Mn^{2+} complex (Nowak and Mildvan, 1972) but also affects the binding of Mn^{2+} to the enzyme-ligand complexes.

A summary of the binding data measured is given in Table I.

Discussion

The cooperative effects of ligand binding to a protein in the presence of a second ligand have been widely observed; however, this phenomenon has usually been qualitatively described in terms of mutual interactions or conformational effects. If the interaction of ligand A to an enzyme is measured by some function f_A, and the interaction is also measured in the presence of ligand B giving $f_{A/B}$, we have a quantitative measure

of the extent of interaction of A. If A interacts with the protein independent of ligand B, $f_A = f_{A/B}$. If the presence of B affects the interaction of A to the protein, then $f_{A/B} - f_A$ is a measure of the extent of interdependence, or cooperativity, which can be either in a positive or a negative sense. In our investigation, we have used the apparent free energy of binding ($\Delta F°$) as a parameter to measure the extent of interaction of the substrates and cations to the enzyme pyruvate kinase. An analogous study measuring the interactions of oxalate and NADH to lactate dehydrogenase has been reported (Kolb and Weber, 1975). Since pyruvate kinase has been well studied in terms of the structures of the enzyme-Mn^{2+}-ligand complexes, it is of interest to attempt to relate the magnitude of cooperative ligand interactions with the structures of the enzyme-metal-ligand complexes.

The binding of the substrate phosphoenolpyruvate was shown to have four binding sites per mole of enzyme via direct binding studies using the technique of Hummel and Dreyer (1962). This value is in contrast to the value of 2.4 reported previously (Reynard et al., 1961) but in accord with a value of 3.8 ± 0.3 reported by Kayne (1971). The value for K_S = 158 μM (Table I) is consistent with the value of K_S required to fit the binding data of phosphoenolpyruvate to the E-Mn^{2+} complex (K_S = 130 μM) (Nowak and Mildvan, 1972), and a factor of 2-3 larger than the values estimated by UV difference spectroscopy (Suelter et al., 1966) and by kinetics (Mildvan and Cohn, 1966), and a factor of 6 lower than the value of K_S assumed to fit other phosphoenolpyruvate binding data by PRR (James et al., 1973).

In the formation of the ternary pyruvate kinase-Mn^{2+}-phosphoenolpyruvate complex, the value of K_3 is virtually the same as that previously reported (Nowak and Mildvan, 1972; James et al., 1973), directly demonstrating that Mn^{2+} does, in fact, increase the binding affinity of the enzyme for the substrate phosphoenolpyruvate by greater than two orders of magnitude, $\Delta(\Delta F°) = -3.13 \pm 0.08$ kcal/mol. From a measure of K_A', it is seen that the binding of Mn^{2+} to the enzyme is enhanced in the presence of phosphoenolpyruvate by a factor of 70, $\Delta(\Delta F°) = -2.48 \pm 0.34$ kcal/mol, demonstrating a reciprocal cooperative effect between Mn^{2+} and phosphoenolpyruvate. The coupling free energies are rather large in a negative direction, indicating a strongly positive cooperative interaction. Weber has estimated (Weber, 1975) that most cooperative interactions will have a coupling free energy between (±) 1-1.5 kcal/mol. Comparing these values with those estimated for other protein systems exhibiting cooperative effects, the Mn^{2+}-phosphoenolpyruvate interaction in pyruvate kinase is the strongest cooperative effect measured to date. One aspect of concern however is the inequality of the $\Delta(\Delta F°)$ values measured. This inequality could be due to either the formation of two different E-M-S complexes, depending upon the path of formation (an explanation not deemed likely), or a sizeable error in one of the thermodynamic determinations. The determination which has the greatest experimental error is the measurement of K_A'. Data is difficult to obtain at low metal occupancy (Figure 4A) because of instrumental sensitivity; thus, a distant extrapolation for analysis is required.

If it is this determination which is in error, an estimate of K_A' from eq 5 or 10 would be 0.2×10^{-6} M, certainly an impossible measurement for us to make with any amount of certainty. The formation of this highly cooperative ternary E-M-S complex results in a large change in the structure of the Mn^{2+}, as measured by EPR (Reed and Cohn, 1973), and the environment of the bound Mn^{2+}, as determined by PRR (Mildvan and Cohn, 1966; Nowak and Mildvan, 1972; James et al., 1973). The structure of this complex has been reported to take place via substrate binding to the Mn^{2+} (Nowak and Mildvan, 1972; Mildvan et al., 1973; James and Cohn, 1974) giving a physical model of the cooperative interaction of these two ligands to the enzyme. Direct coordination to the metal, however, may not be a mandatory prerequisite for cooperativity, since experiments reported with the Co^{2+}-activated enzyme indicate that phosphoenolpyruvate forms a second sphere complex with the metal (Melamud and Mildvan, 1975). The discrepancies between results of the Mn^{2+} and the Co^{2+}-activated enzyme have not yet been resolved in terms of the structure of the E-M-S complex. From optical studies, it is suggested that the activation of pyruvate kinase by Mg^{2+} or Mn^{2+} or by Co^{2+} or Ni^{2+} takes place in a structurally different manner (Kwan et al., 1975). In the formation of the ternary E-M-S complex with ADP, there is little, if any, cooperativity involved; $\Delta(\Delta F°) = -0.08 \pm 0.08$ kcal/mol with only four Mn^{2+} sites per mole. This negligible cooperativity between Mn^{2+} and ADP binding can be compared to the failure of ADP to perturb the ERP spectrum of bound Mn^{2+} (Reed and Cohn, 1973), a lack of change in the circular dichroic (CD) spectrum of the pyruvate kinase-Co^{2+} spectrum (Kwan et al., 1975), and a failure to change the structure of the active site of the enzyme by a measure of the divalent cation-monovalent cation distance (T. Nowak, unpublished observations). This is also consistent with nuclear relaxation studies with the enzyme-Mn^{2+}-ATP complex, which indicates that ADP does not bind in the first coordination sphere of the bound Mn^{2+} (Sloan and Mildvan, 1976).

We have observed that Mn^{2+} and pyruvate also bind in a cooperative manner to pyruvate kinase (Figure 7) with a $\Delta(\Delta F°) = -1.52$ kcal/mol. The extent of cooperativity, although sizeable, is significantly less than that observed with phosphoenolpyruvate. Assuming a K_S for enzyme-pyruvate of $K_S = 1.75 \pm 0.25 \times 10^{-3}$ M based on the values which give a best fit for PRR titration data (T. Nowak, unpublished observations) a $\Delta(\Delta F°) = -1.30 \pm 0.14$ kcal/mol can also be estimated, in agreement with the value obtained for Mn^{2+} binding. These data are consistent with conservation of free energy in this complex and are consistent with CD spectra (Kwan et al., 1975), cation-cation distance data (Nowak, 1977, submitted for publication), and PRR enhancement data (Nowak and Mildvan, 1972), which show a strong interaction between pyruvate and pyruvate kinase-Mn^{2+} complex.

This interaction differs from the interaction of phosphoenolpyruvate with the enzyme-Mn^{2+} complex by these same criteria. A contrast to these criteria is that phosphoenolpyruvate and pyruvate both elicit similar, although not identical, changes in the environment of the bound Mn^{2+} by EPR measurements (Reed and Cohn, 1973). The structure of the enzyme-Mn^{2+}-pyruvate complex does not take place via direct coordination in the primary coordination sphere of Mn^{2+} (Fung et al., 1973), obviating the requirement of direct interaction to elicit a cooperative effect in binding.

The binding of Mn^{2+} in the enzyme-ADP-phosphoenolpyruvate equilibrium complex shows only four cation sites per enzyme. This observation questions a second divalent cation site in the enzyme-nucleotide complex implied from the studies in which the Cr^{III}-ATP complex interacts with the enzyme and serves as a cofactor for the detritiation of pyruvate only in the presence of Mg^{2+} or Mn^{2+} (Gupta et al., 1976). The CrATP complex must serve as an additional cofactor which can act as a phosphate activator for the detritiation reaction (Robinson and Rose, 1972; Rose, 1960); the Cr^{III} atom serves only as a structural factor in the polyphosphate chain and does not have

TABLE II: Coupling Free Energy $\Delta(\Delta F°)$ between Ligands.

Ligand Pair[f]	$\Delta(\Delta F°)$ (kcal/mol)
P-enolpyruvate, Mn^{2+} [a]	-2.48 ± 0.34
	-3.13 ± 0.08
P-enolpyruvate, Mn^{2+} [b]	-1.33 ± 0.12
ADP, Mn^{2+}	-0.08 ± 0.08
Pyruvate, Mn^{2+} [a]	-1.52 ± 0.14
Pyruvate, Mn^{2+} [b]	-1.11 ± 0.08
P-enolpyruvate + ADP, Mn^{2+}	-1.79 ± 0.12
P-enolpyruvate, K^+ [c]	-1.68 ± 0.12
Mn^{2+}, K^+ [d]	-1.26 ± 0.11
Mn^{2+}, $CH_3NH_3^+$ [e]	-0.88 ± 0.11

[a] Calculated for the interactions measured in the presence of K^+. [b] Calculated for the interactions measured in the presence of TMA. [c] Calculated from data taken from Nowak and Mildvan (1972). [d] Calculated from data taken from Suelter et al. (1966) and Nowak and Mildvan (1972). [e] Calculated from data taken from Nowak (1973). [f] Abbreviation used: P-enolpyruvate, phosphoenolpyruvate.

any functional role in terms of catalysis. Cooperativity in terms of Mn^{2+} binding is observed ($\Delta(\Delta F°) = -1.79 \pm 0.12$ kcal/mol) in the catalytic complex. This observation is consistent with the ERP spectrum of Mn^{2+} in the equilibrium complex being anisotropic in contrast with the spectrum of Mn^{2+} in the binary complex (Reed and Cohn, 1973), and the structure of the equilibrium complex is quite compact based on the relative cation distances when this complex is formed (T. Nowak, unpublished observations).

The formation of several of these complexes was also measured in the absence of an activating monovalent cation, and in each case in which $\Delta(\Delta F°)$ was measured (Table II) the extent of cooperativity in ligand binding decreased. Furthermore, in each case where Mn^{2+} binding was compared in the presence of K^+ or of TMA^+ (i.e., Figures 4B, 7B), the stoichiometry of binding in the presence of K^+ is slightly decreased, implying a slight competition between Mn^{2+} and K^+. It has been shown that TMA^+ does not bind to any significant extent to pyruvate kinase (Nowak, 1976). The function of K^+ in this enzyme has been proposed to act as a template for phosphoenolpyruvate binding to elicit the proper conformational structure of the substrate for catalysis (Nowak and Mildvan, 1972) and to allow the proper conformational change of the protein at the active site to occur to allow activation for catalysis (Nowak, 1976).

In summary, we have quantitatively described a strong cooperative interaction in binding to pyruvate kinase of Mn^{2+} and phosphoenolpyruvate and with Mn^{2+} and pyruvate. The interaction of Mn^{2+} and phosphoenolpyruvate is the strongest cooperative interaction estimated (Weber, 1975) and plays a crucial role in the activation of the enzyme for catalysis. The exact nature of the structure of the ternary enzyme-Mn^{2+}-phosphoenolpyruvate has yet to be satisfactorily resolved and would certainly help to explain the mechanisms involved in these cooperative interactions. From the cooperative effects observed between Mn^{2+} and pyruvate, in which the evidence is rather substantial that no direct coordination occurs (Fung et al., 1973) and from cooperative effects calculated for the divalent-monovalent cation interactions (Table II), there is no requirement for direct ligand-ligand interaction to be present for cooperative effects to occur. Cooperative effects can occur by the first ligand inducing a conformational change on the enzyme, affecting the binding site for the second ligand to increase the binding affinity for the second ligand. An analogous effect can be elicited by binding the second ligand, affecting the binding site of the first in a reciprocal fashion. As previously discussed, each of the ligands involved in cooperative ligand binding interactions has been observed to elicit a conformational effect on pyruvate kinase. The substrate ADP has been shown by several criteria not to elicit a conformational change and is not involved in any cooperative ligand binding interactions. Finally, K^+, which has been shown to modulate the conformational changes of the active site required for activation and aids in the binding of phosphoenolpyruvate and pyruvate, plays a strong influence in the cooperative effects occurring in the binding of these ligands.

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pH Dependence of Progesterone Interaction with Progesterone-Binding Globulin. Kinetic and Equilibrium Studies†

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ABSTRACT: The kinetics of binding and dissociation for the progesterone-binding globulin (PBG)·progesterone complex have been measured as a function of pH. The association rate constant appears to be independent of pH from pH 5 to 10 with an average value of $k_{on} = 8.5 \times 10^7\ M^{-1}\ s^{-1}$. The dissociation rate constant is strongly pH dependent with the dependency defined by: $k_{off} = k_0(1 + [H^+]/K_1 + K_2/[H^+])(1 + K_3^*/[H^+])/(1 + K_3/[H^+])$. The best values for the various parameters were $k_0 = 0.0785\ s^{-1}$, $pK_1 = 5.30$, $pK_2 = 10.54$, $pK_3^* = 7.41$, and $pK_3 = 7.21$. Simpler expressions were inadequate to fit the data, and it was concluded that at least three ionizing residues are responsible for the stability of the PBG·progesterone complex. The affinity constant was determined by equilibrium dialysis over the range of pH 3 to 12. The ratio of the association and dissociation rate constants is in agreement with the affinity constant from pH 6.5 to 10.5. The influence of pH on the conformation and binding activity of PBG was also investigated. Denaturation by acid, base, or guanidine hydrochloride leads to a reversible loss of binding activity. Regain of binding activity in all cases is slow with half-times of 0.5 to 2.7 h, depending on conditions. The rate of acid denaturation was determined as a function of pH. The protein was found to be incompletely protonated at pH 1.4, suggesting a buried carboxylic acid residue. The slow renaturation of PBG might be due to the difficulty of burying a charged residue in the protein's interior coupled with steric hindrance by the large carbohydrate moiety of PBG.

A detailed investigation of the physicochemical and steroid binding properties of the progesterone-binding globulin (PBG)[1] of the pregnant guinea pig has been performed in this laboratory (Burton et al., 1974; Stroupe and Westphal, 1975a). This unique high-affinity steroid binder can be obtained in pure form and in appreciable amounts (Cheng et al., 1976). It is a polydisperse glycoprotein with the unusually high carbohydrate content of about 70%, including 17% sialic acid; the polypeptide core has a molecular weight of 27 000. The marked alteration in the intrinsic fluorescence of PBG upon binding steroids (Stroupe et al., 1975) has made possible the determination of the association and dissociation rate constants for complex formation (Stroupe and Westphal, 1975b). Chemical-modification studies have been initiated to identify the amino acids involved in binding (Westphal et al., 1976). To gain additional insight into the amino acid residues responsible for the integrity of the steroid-binding site, we have undertaken a study on the influence of pH on the association and dissociation rate constants. The kinetic results were compared with the affinity constants determined under equilibrium conditions. To aid in the interpretation of these findings, the influence of pH on the conformation of PBG was investigated.

Materials and Methods

PBG was prepared from pooled pregnant guinea pig serum (Grand Island Biologicals Co.) by SP-Sephadex and affinity chromatographies as described by Cheng et al. (1976). Steroids were commercial products; their melting points were verified. [^{3}H]Progesterone was from New England Nuclear; its purity was checked by thin-layer chromatography. Guanidine hydrochloride was Mann "ultrapure". All other chemicals were reagent grade, and water was glass redistilled.

Stopped-Flow Fluorometry. A Gibson-Durrum stopped-flow apparatus equipped with a fluorescence cuvette and connected to a Nova 1200 computer for automatic data collection was used as described previously (Stroupe and Westphal, 1975b). The association and dissociation rates of PBG and progesterone were measured as before (Stroupe and Westphal, 1975b). To avoid errors due to possible differences in concentration, a single unbuffered stock solution of PBG (1.1 μM in 0.1 M NaCl) and a single progesterone solution of the same concentration in 0.1 M NaCl were used in measuring the pH dependence of the association rate. The pH was adjusted prior to loading the samples into the instrument by adding 50

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[1] Abbreviations used are: PBG, progesterone-binding globulin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.