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Thermodynamics of Dimer and Tetramer Formations in Rabbit Muscle Phosphofructokinase†

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ABSTRACT: The self-association of rabbit muscle phosphofructokinase (PFK) was monitored as a function of temperature, pH, and ionic strength in order to understand the thermodynamics of this aggregation process. Thermodynamic parameters obtained from the temperature study show that the dimerization of PFK is characterized by negative entropy and enthalpy changes of -270 ± 5 eu and -87 ± 1 kcal/mol, respectively, with no observable change in heat capacity. This is in contrast to the formation of the tetramer, which is governed by positive entropy and enthalpy changes and a positive heat capacity change of 5000 ± 2000 cal/mol. Low ionic strength also favors the formation of the dimer without a significant influence on the tetramerization, which is enhanced by increasing the pH from 6.00 to 8.55. Furthermore, Wyman linkage analysis [Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–285] reveals that the formation of the tetramer from the monomer between pH 6.00 and pH 8.55 involves the loss of 3.3 protons. Further analysis shows that ionization of residues with an apparent pK_a of 6.9 is linked to the formation of PFK tetramers. The conclusion of this study indicates that the major noncovalent forces governing the formation of the dimer are different from those for the association of the tetramer.

The self-association of rabbit muscle phosphofructokinase (PFK)[1] has been the topic of many investigations by a variety of techniques (Parmeggiani et al., 1966; Ling et al., 1965; Leonard & Walker, 1972; Pavelich & Hammes, 1972; Goldhammer & Paradies, 1979; Hesterberg & Lee, 1981, 1982; Luther et al., 1983, 1985). One of the goals for such intensive investigation is to elucidate the role of self-association in the regulation of enzyme activity. Recently, there is an increasing number of reports to indicate that the kinetic properties of PFK are dependent on protein concentrations (Reinhart, 1984; Luther et al., 1985). Hence, an elucidation of the mechanism of regulation of PFK activity is linked to a knowledge on the basic thermodynamics that govern the self-association of PFK.

Luther et al. (1985) showed that, within the limits of resolution by sedimentation velocity, *active* PFK undergoes reversible self-association and the various oligomeric species are in a rapid, dynamic equilibrium. In conjunction with other reports from the literature and this laboratory, it was shown

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[1] Abbreviations: PFK, phosphofructokinase; 3× TEMA buffer, 75 mM Tris–carbonate, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA; IEMA buffer, 75 mM imidazole, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA at pH 7.0; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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that at pH 7.0 the mode of association is best described as $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. Since the thermodynamic parameters governing this equilibrium are not available, the self-association of PFK was determined as a function of temperature, pH, and ionic strength. Results from this study show that the formations of the dimeric and tetrameric species of PFK involve different predominant driving forces.

Experimental Procedures

Materials. Tris–carbonate, $MgCl_2$, and EDTA were obtained from Sigma Chemical Co. Imidazole and $(NH_4)_2SO_4$ were purchased from Boehringer-Mannheim and Schwarz/Mann, respectively.

PFK was purified, assayed, and stored as previously described (Luther et al., 1985). In all experiments, PFK was equilibrated in the appropriate buffer by passage through a Sephadex G-25 column (1.2 × 8.5 cm).

Methods. Sedimentation velocity studies were conducted, and the results were analyzed by procedures previously published (Hesterberg & Lee, 1981).

The pH study was conducted in 75 mM Tris–carbonate, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA (3× TEMA) as the indicated pH (between pH 7.0 and pH 8.55) and 23 °C. For the pH range between 6.0 and 6.7, IEMA buffer was employed. The buffer for the ionic strength study was identical with that used in the pH studies. Ionic strength was varied by changing the concentration of Tris–carbonate.

The temperature study was performed in 75 mM imidazole, 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA (IEMA) at pH 7.0, as a function of temperature between 5 and 23 °C. The pH values were measured and adjusted at the temperature of study. Control experiments reveal that the substitution of imidazole for Tris–carbonate does not alter the self-association of PFK.

Results

Before a successful analysis of sedimentation velocity data can be conducted, the association–dissociation reaction must be shown to exist in a rapid, dynamic equilibrium. Criteria used previously in this laboratory were employed, i.e., to determine the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, as a function of rotor speed and to monitor $\bar{s}_{20,w}$ of diluted PFK samples as a function of time of dilution from a concentrated stock solution. Results show that the value of $\bar{s}_{20,w}$ exhibits no dependence on rotor speed or time of dilution; thus, it can be concluded that under all experimental conditions employed in this study PFK exists in a rapid, dynamic equilibrium. The concentration dependence of the sedimentation behavior of PFK as a function of temperature is shown in Figure 1. With decreasing temperatures, a decrease in the values for the sedimentation coefficient is observed. This indicates a shift in the association–dissociation equilibrium toward the formation of smaller species. The data were further analyzed by curve fitting procedures previously used in this laboratory (Hesterberg & Lee, 1981). Briefly, the relation between $\bar{s}_{20,w}$ and protein concentration is

$$\bar{s} = \sum_i s_i^\circ (1 - g_i C) K_i C_1^i / \sum_i K_i C_1^i \qquad (1)$$

where K_i is the equilibrium constant between any $i$mer and the monomer, C_1 is the monomer concentration, $C = \sum_i K_i C_1^i$, g_i is the nonideality coefficient, and s_i° is the sedimentation coefficient of the ith species at infinite dilution. The various s_i° values used in the fitting are the same as those employed previously (Hesterberg & Lee, 1981), namely, $s_1^\circ = 4.95$ S, $s_2^\circ = 7.6$ S, $s_4^\circ = 13.5$ S, $s_8^\circ = 19.7$ S, and $s_{16}^\circ = 34.0$ S. In all cases, if s_4° is assumed to be 12.4 S, significantly worse fits are obtained. This is basically because the buffer components do not include substrates, which have been shown to convert the 13.5S form to one of 12.4 S (Hesterberg et al., 1981; Luther et al., 1983). The data were analyzed by a variety of reaction schemes of different stoichiometry. The values of equilibrium constants are tabulated and summarized in Table I. It is evident that under certain conditions more than one mode can describe the data equally well. Since the goal of this investigation is to find the simplest model that best describes the data, the following set of rules were established to assess the reliability of data analysis:

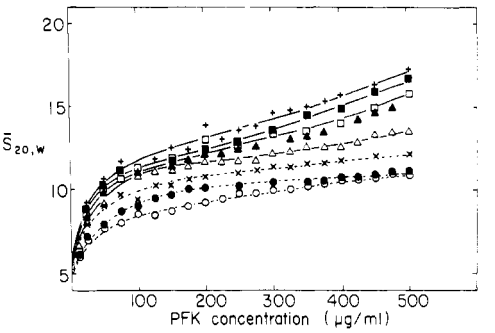

FIGURE 1: Effect of temperature on concentration dependence of weight-average sedimentation coefficient, $\bar{s}_{20,w}$, in IEMA buffer at pH 7.0. Symbols for the corresponding temperature at which the study was conducted are (○) 5, (●) 6, (×) 10, (△) 12.5, (▲) 15.0, (□) 17.5, (■) 20, and (+) 23 °C. The lines represent the theoretical fit of the experimental data with the association model of $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons \mu_8$ for the results at 5 and 10 °C and $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ for the results at 12.5–23 °C. The data points represent the average of multiple data sets.

(1) Under one experimental condition, select that mode of association which has the lowest value of the standard root mean square deviation σ, which is a measure of how well the calculated data compare with the experimental data (Hesterberg & Lee, 1981).

(2) If two or more modes of association have equal or very similar σ values, then the mode with the smallest number of species is chosen.

(3) If rules 1 and 2 cannot allow differentiation between modes of association, then only those equilibria with consistent values for apparent association constants K_i^{app} are included in the final data analysis.

In application of these rules, it can be concluded that the only values that can be used with reasonable confidence are those for the dimer and tetramer.

From 5 to 15 °C, it is apparent from the rules stated that the data must include at least a monomer, dimer, and tetramer. The physical existence of these species has been verified by various techniques, including chemical cross-linking and sedimentation (Lad & Hammes, 1974; Telford et al., 1975; Paradies & Vetterman, 1975; Paradies, 1979; Hesterberg et al., 1981; Luther et al., 1983). Table I reveals that the major effect of temperature is on K_2^{app}. This value decreases with increasing temperature, such that at 23 °C, pH 7.0 in IEMA, the dimer has little or no effect on the self-association of PFK. The observed changes for the apparent tetramerization constant K_4^{app} are not the same as those K_2^{app}. From 5 to 12.5 °C, the K_4^{app} value decreases with an increase in temperature, while from 15 to 23 °C the K_4^{app} value increases. Results of this study suggest that these two processes may be governed by different thermodynamic parameters.

In order to further define the effect of temperature on the self-association of PFK, a determination of the thermodynamic parameters for this process was conducted. Panels A and B of Figure 2 plots for k_2^{app} and k_4^{app} as a function of temperature.

Table I: Summary of Self-Association Studies on PFK as a Function of Temperature in 3× IEMA Buffer at pH 7.0[a]

T (°C)	stoichiometry	s_4°	K_2^{app} (mL/mg)	K_4^{app} (mL/mg)^3	K_8^{app} (mL/mg)^7	K_{16}^{app} (mL/mg)^{15}	σ
5	1-2-4	13.5	324	1.63×10^6			1.10
	1-4-16			2.9×10^3		2.3×10^{15}	3.97
	1-2-4-8		*328*	*1.68×10^6*	6.06×10^{11}		0.07
	1-2-4-16		328	1.61×10^6		6.22×10^{24}	0.2
	1-2-4-8-16		306	1.40×10^6	3.22×10^{11}	1.33×10^{23}	0.03
6	1-4-16	13.5		2.31×10^4		1.05×10^9	7.32
	1-2-4-8		*140*	*8.60×10^5*	2.03×10^{10}		0.06
	1-2-4-16		140	8.62×10^5		4.13×10^{22}	0.32
	1-2-4-8-16		105	5.81×10^5	6.95×10^9	5.05×10^{21}	0.08
10	1-4-16	13.5		1.52×10^5		7.18×10^9	1.98
	1-2-4-8		*56.0*	*7.14×10^5*	9.00×10^{10}		0.32
	1-2-4-16		65.0	6.30×10^5		1.31×10^{23}	0.48
	1-2-4-8-16		52.5	4.41×10^5	4.95×10^{10}	1.00×10^{21}	0.21
12.5	1-4-16	13.5		3.62×10^5		2.11×10^{22}	0.18
	1-2-4-8		1.05	3.26×10^5	5.99×10^{10}		0.73
	1-2-4-16		5.16	6.35×10^5		2.03 × 1023	0.12
	1-2-4-8-16		*8.44*	*3.45×10^5*	2.90×10^{10}	2.11×10^{22}	0.07
15	1-4-16	13.5		7.60×10^5		7.75×10^{23}	0.29
	1-2-4-8		1.18	7.60×10^5	4.95×10^{11}		1.25
	1-2-4-16		0.71	7.13×10^5		1.00×10^{24}	0.16
	1-2-4-8-16		*0.14*	*6.85×10^5*	1.90×10^8	2.11×10^{22}	0.07
17.5	1-4-16	13.5		*9.2×10^5*		2.88×10^{24}	0.21
	1-2-4-8		8.44	8.41×10^5	1.00×10^{12}		1.10
	1-2-4-16		0.37	9.10×10^5		2.83×10^{24}	0.23
	1-2-4-8-16		0.16	8.50×10^5	2.31×10^{11}	1.13×10^{24}	0.09
20	1-4-16	13.5		*1.05×10^6*		7.05×10^{24}	0.20
	1-2-4-8		0.23	9.12×10^5	1.93×10^{12}		5.77
	1-2-4-16		0.10	1.40×10^6		6.02×10^{24}	0.22
	1-2-4-8-16		0.23	1.10×10^6	4.95×10^{11}	9.26×10^{24}	
23	1-2-4	13.5	1.25	3.50×10^6			1.25
	1-4-16			*1.30×10^6*		2.20×10^{25}	0.12
	1-2-4-8		1.10	1.20×10^6	6.55×10^{12}		5.30
	1-2-4-16		0.30	3.60×10^6		6.82×10^{25}	0.08
	1-2-4-8-16		0.04	1.43×10^6	7.7×10^{10}	2.90×10^{25}	0.10

[a] Italicized values are those used in Figure 2.

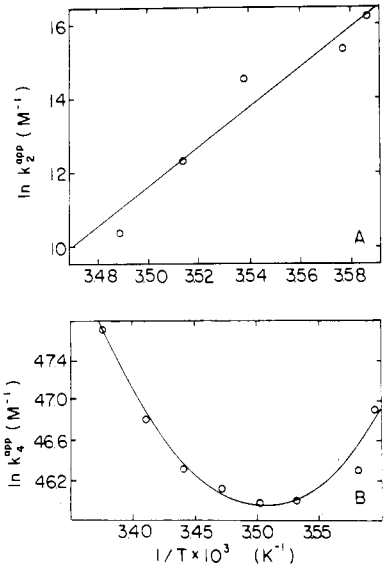

FIGURE 2: Relations between equilibrium constants and temperature for self-association of PFK in IEMA buffer at pH 7.00: (A) dimerization and (B) tetramerization reactions. k_i^{app} is the apparent molar association constant for the indicated species i. Temperature is expressed in Kelvin. The solid lines represent the respective relations with the parameters shown in Tables II and III.

The equilibrium constants in Figure 2 are reported in units of M^{-1} and are related to the values reported in Table I by

$$k_i = K_i M_1 / i \quad (2)$$

where M_1 is the monomer molecular weight of 83 000.

The temperature dependence plot for the tetramer formation displays pronounced curvature; therefore, the data were fit to

$$\ln k_i^{app} = a + b(1/T) + c \ln T \quad (3)$$

Table II: Thermodynamic Parameters for Dimerization of PFK in IEMA Buffer at pH 7.0

T (°C)	k_2^{app} (L/mol)	ΔG°_{obsd} (kcal/mol)	ΔH°_{obsd} (kcal/mol)	ΔS°_{obsd} (eu)	ΔC_p [cal/(mol·deg)]
5	1.36×10^7	−9.1 ± 0.1	−88 ± 1	−288 ± 5	0
6	5.60×10^6	−8.6 ± 0.1	−88 ± 1	−278 ± 5	
10	2.31×10^6	−8.2 ± 0.1	−87 ± 1	−278 ± 5	
12.5	2.14×10^5	−7.0 ± 0.1	−87 ± 1	−278 ± 5	
15		−5.5 ± 0.1	−86 ± 1	−278 ± 5	

which is a truncated form of the integrated van't Hoff equation (Glasstone, 1947). The values of the changes in free energy ΔG°, enthalpy ΔH°, entropy ΔS°, and heat capacity ΔC_p for the tetramerization of PFK are given by

$$\Delta G^\circ = -RT \ln k_i^{app}; \quad \Delta H^\circ = R(cT - b)$$
$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T; \quad \Delta C_p = Rc \quad (4)$$

The formation of the PFK dimer at temperatures between 5 and 10 °C is characterized by negative enthalpy and entropy changes and no heat capacity change, as summarized in Table II. The thermodynamic parameters that govern the tetramerization of PFK are summarized in Table III. These results are quite different from that of the dimerization reaction. Tetramer formation is characterized by increasing positive values of ΔH° and ΔS° with increasing temperature. The reaction is further characterized by a positive heat capacity change of 5000 cal/(mol·deg). This indicates that the predominant driving forces for the dimerization and tetramerization reactions are apparently different and separate contact surfaces are involved.

The effect of ionic strength on the self-association of PFK was determined by varying the Tris–carbonate concentration and keeping the other buffer components constant. Results

Table III: Thermodynamic Parameter for Tetramerization of PFK in IEMA Buffer at pH 7.00

T (°C)	k_4^{app} (L/mol)	ΔG°_{obsd} (kcal/mol)	ΔH°_{obsd} (kcal/mol)	ΔS°_{obsd} (eu)	ΔC_p [cal/(mol·deg)]
5	2.29×10^{20}	−25.9 ± 0.1	−36 ± 0.5	−20 ± 3.0	
6	1.20×10^{20}	−26.1 ± 0.1	−30 ± 0.5	2 ± 3.0	5000 ± 2000
10	1.02×10^{20}	−26.2 ± 0.1	−10 ± 0.5	74 ± 3.0	
12.5	9.1×10^{19}	−26.1 ± 0.1	−3 ± 0.5	120 ± 3.0	
15	1.70×10^{20}	−26.4 ± 0.1	15 ± 0.5	160 ± 3.0	
17.5	1.30×10^{20}	−26.7 ± 0.1	28 ± 0.5	204 ± 3.0	
20	2.01×10^{20}	−27.3 ± 0.1	41 ± 0.5	225 ± 3.0	
23	5.0×10^{20}	−28.1 ± 0.1	56 ± 0.5	300 ± 3.0	

Table IV: Summary of Self-Association Studies as a Function of Ionic Strength at pH 7.0 and 23 °C

concn of buffer (mM)	ionic strength (M)	stoichiometry	S_4°	K_2^{app} (mL/mg)	K_4^{app} (mL/mg)^3	K_8^{app} (mL/mg)^7	K_{16}^{app} (mL/mg)^{15}	σ
5	0.105	1-4-16	13.5		7.03×10^5		5.23×10^{23}	1.53
		1-2-4-8		25.0	5.30×10^5	5.05×10^{11}		0.09
		1-2-4-16		11.0	5.30×10^5		1.30×10^{22}	0.75
25	0.153	1-4-16	13.5		9.90×10^5		3.61×10^{24}	0.09
		1-2-4-8		21.0	7.00×10^5	9.89×10^{11}		2.62
		1-2-4-16		2.25	1.70×10^6		3.21×10^{25}	0.11
75	0.278	1-4-16	13.5		1.30×10^6		2.21×10^{25}	0.03
		1-2-4-8		1.10	1.20×10^6	6.55×10^{12}		5.30
		1-2-4-16		0.38	5.20×10^6		5.4×10^{25}	0.04

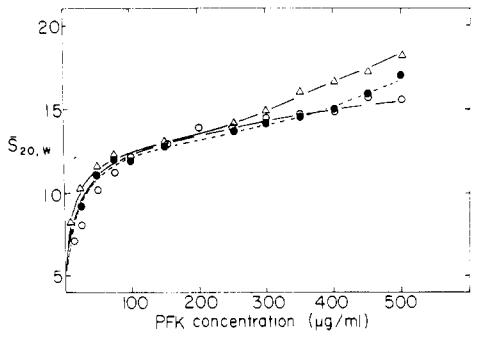

FIGURE 3: Effect of ionic strength on concentration dependence of weight-average sedimentation coefficient of PFK at pH 7.0 and 23 °C. Studies were performed in (○) 5, (●) 25, or (△) 75 mM Tris–carbonate in 18 mM $MgCl_2$, 9 mM $(NH_4)_2SO_4$, and 3 mM EDTA.

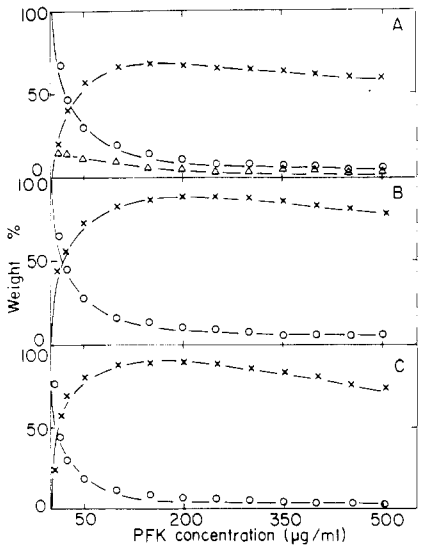

FIGURE 4: Weight distribution of various polymeric species of PFK at pH 7.0 and 23 °C as a function of ionic strength. The calculations are based on a self-associating system of (A) $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_8$ and (B and C) $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ as a function of PFK concentration. The ionic strength and corresponding figures are (A) I = 0.105 M, (B) I = 0.153 M, and (C) I = 0.278 M. Apparent equilibrium constants for each condition are listed in Table IV. Symbols are as follows: (○) monomer, (△) dimer, and (×) tetramer.

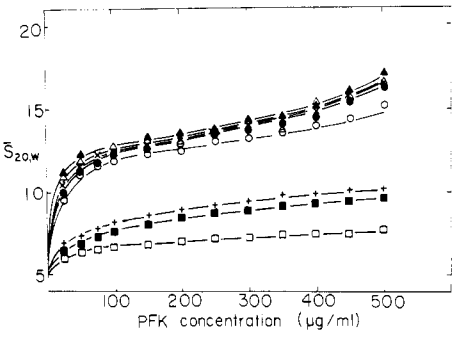

FIGURE 5: Effect of pH on concentration dependence of weight-average sedimentation coefficient, $\bar{s}_{20,w}$, of PFK at 23 °C. Symbols and corresponding pH are (□) 6.0, (■) 6.5, (+) 6.7, (○) 7.0, (●) 7.3, (×) 7.5, (△) 8.0, and (▲) 8.55. The solid lines represent the theoretical fit of the experimental data with the association model $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ and a s_4° of 13.5 S. The data points represent the average of multiple data sets. The buffer for the pH range of 6.0–6.7 was IEMA whereas 3× TEMA was employed in the other pH conditions.

shown in Figure 3 reveal that, as the ionic strength of the buffer is decreased, the values of $\bar{s}$ at specific protein concentration are also decreased. Quantitative analysis of the data is summarized in Table IV. Application of the rules described for the determination of the mode of self-association reveals that at low ionic strength (I = 0.095 M) the simplest model that best fits the data is $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4 \rightleftharpoons M_8$. When the ionic strength is increased, no significant amounts of dimer and octomer are present, and the stoichiometry is now best described as $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$. The largest and most consistent effect is seen on the tetrameric form. Weight-distribution plots shown in Figure 4 reveal that with increasing ionic strength the amount of tetramer present at low protein concentration increases. The presence of dimer is only significant at the lowest ionic strength. This suggests that by increasing the ionic strength electrostatic charges that favor the formation of the dimer are screened, thus weakening the dimerization constant. However, the formation of the tetramer is enhanced. These results are consistent with that from the temperature-dependence study; i.e., the formation of dimer and tetramer involves different surfaces of contact.

The effect of charges on the association–dissociation of PFK was further investigated by examining the self-association as a function of H^+ concentration. Figure 5 shows the effect of

Table V: Summary of Self-Association Studies on PFK as a Function of pH at 23 °C

pH	stoichiometry	S°_4	K_2^{app} (mL/mg)	K_4^{app} (mL/mg)^3	K_{16}^{app} (mL/mg)^{15}	σ
6.0	1–2–4	13.5	57	1.25×10^3		0.028
	1–4–16			2.18×10^4	6.93×10^{16}	187
	1–2–4–16		93	1.60×10^3	5.80×10^{16}	0.10
6.5	1–2–4	13.5	200	5.10×10^4		0.029
	1–4–16			2.00×10^3	5.00×10^{17}	21.8
	1–2–4–16		189	8.20×10^4	4.30×10^{20}	0.18
6.7	1–2–4	13.5	337	2.51×10^5		0.032
	1–4–16			3.99×10^5	4.94×10^{21}	120
	1–2–4–16		220	2.00×10^5	3.74×10^{21}	0.05
7.0	1–2–4	13.5	0.54	2.23×10^5		48
	1–4–16			1.30×10^6	2.21×10^{25}	0.03
	1–2–4–16		0.17	1.80×10^6	5.5×10^{25}	0.28
7.3	1–2–4	13.5	0.25	2.73×10^6		0.24
	1–4–16			2.00×10^6	4.63×10^{25}	0.07
	1–2–4–16		0.11	2.71×10^6	1.59×10^{26}	0.11
7.5	1–4–16	13.5		2.99×10^6	1.89×10^{26}	0.09
	1–2–4–16		0.21	3.80×10^6	6.16×10^{26}	0.18
8.0	1–4–16	13.5		4.00×10^6	4.83×10^{26}	0.24
	1–2–4–16		0.02	5.21×10^6	1.43×10^{27}	0.06
8.55	1–4–16	13.5		8.30×10^6	1.46×10^{28}	0.14
	1–2–4–16		0.02	9.53×10^6	2.51×10^{28}	0.30

varying pH between 6.00 and 8.55 at 23 °C on the self-association of PFK. These results reveal that the association of PFK is enhanced at lower H^+ concentrations (i.e., increased pH). Quantitative analysis of the data, as summarized in Table V, reveals that the simplest model that best describes the data is $M_1 \rightleftharpoons M_4 \rightleftharpoons M_{16}$ at pH between 7.00 and 8.55, with s°_4 assuming a value of 13.5S, while the simplest model that best describes the data at pH lower than 7.00 is $M_1 \rightleftharpoons M_2 \rightleftharpoons M_4$. The weight percentage of tetramer is lower as pH decreases from 6.70 to 6.00. This coincides with the loss of the activity of PFK from pH 6.70 down to pH 6.00.[2]

Since the quantitative data obtained are a reflection of the thermodynamic linkage of proton binding and self-association, the data were analyzed in terms of the linked-function relations developed by Wyman (1964):

$$\left(\frac{\partial \ln k_4^{app}}{\partial \ln a_{H^+}}\right)_{T,P,a_j} = \bar{\nu}_{4,H^+} - 4\bar{\nu}_{1,H^+} = \Delta\bar{\nu}_{H^+} \qquad (5)$$

where $\bar{\nu}_{4,H^+}$ and $\bar{\nu}_{1,H^+}$ are the number of protons per tetramer and monomer, respectively, and $\Delta\bar{\nu}_{H^+}$ is the change in the number of protons during the formation of a tetramer from a monomer. An analysis of the results in Table V by this linkage yielded a value of −3.3 for $\Delta\bar{\nu}_{H^+}$. This number does not need to be and generally is not an integral value and as stated represents the changes in the number of protons binding upon formation of a tetramer. The simplest interpretation is, therefore, that upon formation of a tetramer one ionizable group in each monomer has to be deprotonated.

Discussion

The approach used in this study was to quantitatively determine the effects of pH, temperature, and ionic strength on the self-association of PFK and then use this information to determine the thermodynamics of this association–dissociation reaction. The thermodynamic parameters obtained from this investigation reveal that the formation of the dimer is characterized by negative enthalpy and negative entropy changes with no apparent heat capacity change. This is in contrast to results for the formation of the PFK tetramer, which display a positive entropy and enthalpy change with a large positive heat capacity change. These results imply that the forces that predominate in the formation of the dimer are different from those that control the assembly of the tetramer. Dimerization is probably involved in ionic interaction whereas the tetramerization is driven by hydrophobic interaction.

Previous studies on native and cross-linked PFK using such techniques as electron microscopy and small-angle X-ray scattering have concluded that dimers consist of monomers lying side-by-side and that tetramers are formed by two dimers in a planar end-to-end array (Telford et al., 1975; Paradies & Vetterman, 1976; Paradies, 1979). These results, however, are in contrast to others that suggest D_2 symmetry for the tetramer with each subunit located at the tip of a tetrahedral structure (Hesterberg et al., 1981; Poorman et al., 1984). Although different models for the tetramer are predicted, these studies all suggest that different surfaces will be involved in the formation of the tetramer in comparison with the dimer. However, the basis for these conclusions is on gross structural studies and in no way defines any of the interactions or the thermodynamics for these associations. Furthermore, in a recent report the primary structure of rabbit muscle PFK (Poorman et al., 1984) indicated that there is a large degree of homology with that of the *Bacillus stearotheromophillus* (BS) PFK, a prokaryotic enzyme whose three-dimensional crystallographic structure is known (Kolb et al., 1980; Evans et al., 1981). Although the apparent molecular weights of the subunits are different, the dimer of BS PFK shows a great amount of symmetry with the amino-terminal and carboxy-terminal halves of the subunit for rabbit muscle PFK. Using the known crystallographic information for BS PFK, Poorman et al. (1984) propose a model for the formation of the tetramer. From their model, it is obvious that the formation of the dimer and tetramer is along different axis, further suggesting that different interactions may be involved in the formation of the dimers and the tetramer.

While it is apparent that different forces control the formation of the dimer and tetramer, the exact nature of these forces is still not clear. In considering the known structural changes that occur upon the association–dissociation of proteins, it is evident that a wide variety of different types of interactions may be simultaneously involved in contributing to the observed thermodynamic parameters (Timasheff, 1973; Sturtevant, 1977; Ross & Subramarian, 1981). A detailed accounting of such effects must include energies that arise as a result of variations in van der Waals contacts, hydrogen bonds, salt bridges, electrostatic, and hydrophobic interactions. The thermodynamic effects from the motional dynamics of

[2] G.-Z. Cai and J. C. Lee, unpublished results.

the molecules in their various states of association must also be considered. Therefore, the observed enthalpies and entropies are the result of the contribution from many large terms, which may or may not cancel each other. Ackers has pointed out that from the observed energetics it is not possible to determine which noncovalent interaction(s) is (are) contributing significantly (Ackers, 1980). However, on the basis of results presented in this study, it is clear that different types of noncovalent interactions play dominant roles in the assembly of the dimer in comparison with the formation of the tetramer. The characteristics of the thermodynamic parameters associated with the tetramer formation, i.e., positive $\Delta H°$ and $\Delta S°$, and increased association at higher ionic strength imply a dominant role of hydrophobic interactions in the association of tetramers. The negative enthalpy and entropy and increased formation of dimer at low ionic strength and pH are consistent with an increased role of ionic and/or van der Waals forces in the formation of the dimer. However, until crystallographic data on the three-dimensional structure of PFK are available, such that the areas of contact between subunits can be verified, no specific conjectures can be made on the interactions involved.

On the basis of the pH dependence study, the difference in the number of protons involved in PFK tetramerization could either signify the titration of a specific group(s) or merely be a general change in the redistribution of protons bound to PFK that results from a change in the electrostatic free energy of the enzyme as it undergoes association from a monomer to a tetramer. This change may also reflect conformational changes. In the presence of substrates, either alone or together, the PFK tetramer is known to undergo a conformational change from an active 13.5S tetramer to an active 12.4S form (Hesterberg et al., 1981; Luther et al., 1983). All the experiments presented in this study are performed in the absence of substrates, and the conclusions from the modeling of the data as a function of pH indicate that all the data are best fit when one assumes the tetramer to have a sedimentation coefficient, $s°_4$, of 13.5 S, i.e., that of the inactive form. If other values for the tetramer are used, e.g., 12.4 S or 13.0 S, the data do not fit well. This suggests that a major conformational change has not occurred; however, it does not eliminate the possibility of small changes that are not detectable by these approaches.

Assuming that no conformational changes have occurred in PFK as a function of pH, the simplest model for the linkage of protonation/deprotonation with the formation of the tetramer from the monomer is given by

$$\begin{array}{ccc} 4M & \rightleftharpoons & M_4 \\ 4H^+ \uparrow\downarrow & & \uparrow\downarrow 4H^+ \\ 4MH^+ & & \end{array} \qquad (6)$$

where M_4 represents the tetramer and M and MH^+ are the unprotonated and protonated forms of the monomer. The linkage of protonation/deprotonation with the assembly of PFK can be mathematically described as

$$\ln k^{app}_{4,obsd} = \ln k^{app}_{4,H=0} - 4 \ln (1 + [H^+]/K^{app}_{m,H^+}) \qquad (7)$$

where $k^{app}_{4,obsd}$ is the observed apparent tetramerization constant at the applicable pH, $k^{app}_{4,H=0}$ represents the apparent association for tetramer formation in the absence of proton binding, and K^{app}_{m,H^+} refers to the apparent proton dissociation constant. This equation states that the observed equilibrium will be composed of contributions from the assembly of the tetramer from the monomer and those from proton dissociation. Nonlinear least-squares analysis of eq 7 was employed to obtain values

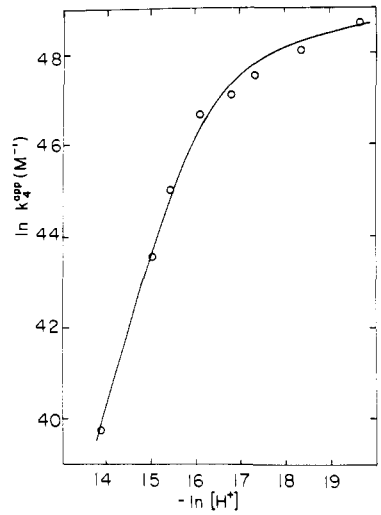

FIGURE 6: Dependence of apparent association constant on hydrogen ion concentration. The buffer is 3× TEMA with H^+ as the variable at 23 °C. Open circles are experimental results, and the solid line represents the nonlinear least-squares fit of the data according to eq 7 with the values of $k^{app}_{4,H^+=0} = 1.4 \times 10^{21}$ M^{-1} and $K^{app}_{m,H^+} = 1.2 \times 10^{-7}$ M.

for $k^{app}_{4,H^+=0}$ and K^{app}_{m,H^+}. The values are $(1.4 \pm 0.2) \times 10^{21}$ M^{-1} and $(1.3 \pm 0.3) \times 10^{-7}$ M for $k^{app}_{4,H^+=0}$ and K^{app}_{m,H^+}, respectively, and the solid line in Figure 6 is a representation of these parameters.

The values obtained from this analysis can be used to determine what effects protonation plays in the linkage scheme. The observed $\Delta G°_{4,obsd}$ for the reaction, as stated, will be made up of contributions from the protonation/deprotonation of PFK and the self-assembly of the tetramer from the monomer as shown in eq 8. Thus, from the linkage scheme, the observed free energy change for tetramerization equals

$$\Delta G^{app}_{4,obsd} = \Delta G^{app}_{4,H=0} + \Delta G°_n \qquad (8)$$

where $\Delta G°_{4,obsd}$ is the observed free energy change at a given pH, $\Delta G°_{4H^+=0}$ is the free energy change for the association of tetramers, without contributions from protons (i.e., at zero proton binding), and $\Delta G°_n$ is the free energy change for the release of protons from the monomer. $\Delta G°_{4H^+=0}$ can be calculated from the reaction in eq 7 and is equal to -28.6 ± 0.1 kcal/mol. Applying this value to eq 8, one finds that $\Delta G°_n$ has a value of 1.14 ± 0.2 kcal/mol. The conclusion from this analysis is that protonation of PFK increases the value of $\Delta G°_{4,obsd}$, thus shifting the equilibrium toward dissociation. Furthermore, this analysis reveals that the dissociation constant of protons from the monomer (K_{m,H^+}) of $(1.3 \pm 0.3) \times 10^{-7}$ M corresponds to pK of 6.9 ± 0.1, indicating a group(s) of that pK which is (are) apparently important in the tetramerization of PFK.

The results of this investigation establish a thermodynamic boundary for which the association–dissociation of PFK must abide. For the range of PFK concentration, it is clear that the dimer and tetramer have major roles in this equilibrium and are controlled by different interactions. However, the energetics determined from this study cannot solely determine the mechanism for the association–dissociation of PFK. This can only be deciphered through the application of this thermodynamic informaton to other data on the structural properties of PFK.

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A Protein Kinase Copurified with Chick Oviduct Progesterone Receptor†

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ABSTRACT: A magnesium-dependent protein kinase activity was copurified with both the molybdate-stabilized 8S form of the chick oviduct progesterone receptor (PR) and its B subunit. In each case, purification was performed by hormonal affinity chromatography followed by ion-exchange chromatography. The K_m(app) values of the phosphorylation reaction for [γ-^{32}P]ATP and calf thymus histones were $\sim 1.3 \times 10^{-5}$ M and $\sim 1.6 \times 10^{-5}$ M, respectively, and only phosphorylated serine residues were found in protein substrates, including PR B subunit. Physicochemical parameters of the enzyme [p$I \sim 5.3$, Stokes radius ~ 7.2 nm, sedimentation coefficient ($s_{20,w}$) ~ 5.6 S, and $M_r \sim 200\,000$] were compared to those of purified forms of PR (B subunit, p$I \sim 5.3$, Stokes radius ~ 6.1 nm, and $M_r \sim 110\,000$; 8S form, Stokes radius ~ 7.7 nm and $M_r \sim 240\,000$). The results suggest that most of the protein kinase activity copurified with both oligomeric and monomeric forms of PR belongs to an enzyme distinct from currently known receptor components. Its physiological significance remains unknown.

Steroid receptors are phosphoproteins (Housley & Pratt, 1983; Grandics et al., 1984; Dougherty et al., 1982, 1984). Steroid receptor phosphorylation may be involved in the modulation of hormone binding (Migliaccio et al., 1982), in the change of receptor conformation (Housley et al., 1982), and in receptor transformation or activation (Barnett et al., 1980; Logeat et al., 1985; Garcia et al., 1986). In vitro phosphorylation of purified chick oviduct progesterone receptor (PR) by endogenous (Garcia et al., 1983) or exogenous (Weigel et al., 1981; Ghosh-Dastidar et al., 1984) protein kinases has been reported. However, the specific enzymes regulating the phosphorylation of steroid receptors in vivo are still unknown.

We have previously reported the copurification of two distinct protein kinase activities associated with purified chick oviduct PR preparations, corresponding to the B subunit of M_r 110 000 and to the 8S PR complex, respectively (Garcia et al., 1983). The B subunit preparation displayed Mg^{2+}-dependent activity and phosphorylated contaminants and histone substrates, as well as the B subunit itself. No effort was made to separate protein kinase activity from the PR B subunit. In the 8S PR extract, another "protein kinase activity", observed only in the presence of a high concentration of calcium, was described. It labeled specifically the hsp 90 component (Catelli et al., 1985), as observed after electrophoresis; however, subsequently we found that this labeling

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