Reversible Dimerization of Avian Pancreatic Polypeptide†

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ABSTRACT: The dimerization of avian pancreatic polypeptide (PP) was studied by large-zone gel chromatography using integral boundary analysis. The association constant has been determined as a function of temperature and pH. The dimerization is endothermic and entropically driven, which suggests hydrophobic interactions, and is enhanced with increasing pH. Analysis of the pH dependence indicates the involvement of ionizable groups, with pK_a values of 4.5–5.5. In the avian PP molecule, there are six groups which are potentially titratable in this pH range. A comparison of the amino acid sequence of avian PP with that of the bovine and canine homologues, which also exhibit pH-dependent dimerization [Noelken, M. E., Chang, P. J., & Kimmel, J. R. (1980) *Biochemistry* (preceding paper in this issue)], shows that they have three carboxylate and two guanidinium groups in common. It is suggested that salt linkages involving these groups participate in dimerization. In the avian peptide, histidine-34 may also be involved.

Pancreatic polypeptide (PP)[1] from avian, bovine, and canine sources has been demonstrated to have significant α-helix content in solution and to undergo a pH-dependent association (Noelken et al., 1980). In the case of the avian hormone, this association involves a monomer–dimer equilibrium, and it is possible that the hydrophobic face of the α helix predicted for avian PP is involved in this dimer formation. Indeed, there is ample precedent for this type of association in two other pancreatic hormones, glucagon and insulin (Sasaki et al., 1975; Pullen et al., 1976), and the peptide segments involved in association of these hormones are thought to represent the faces which interact with the respective receptors. This study was undertaken to obtain information about the driving forces involved in avian PP association and to define the thermodynamic parameters involved.

The method of integral boundary analysis using large-zone molecular sieve chromatography (Ackers & Thompson, 1965) was chosen for study of the association equilibrium. The usefulness of this method in the analysis of associating systems was first suggested by Winzor & Scheraga (1963), who demonstrated that the mass transport theories developed by Gilbert (1955) for sedimentation and electrophoresis were applicable to gel chromatography. Analogous transport equations were subsequently derived specifically for gel chromatography by Ackers & Thompson (1965). The use of gel chromatography is relatively rapid, simple, and inexpensive, yet yields good accuracy and sensitivity. Various parameters, such as temperature, pH, and ionic strength, may be altered easily. This approach has been exploited to study the association equilibria of various proteins, including α-chymotrypsin (Winzor & Scheraga, 1963), carboxyhemoglobin (Ackers & Thompson, 1965), D-amino acid oxidase apoenzyme (Henn & Ackers, 1969), and hemerythrin (Rao & Keresztes-Nagy, 1972; Tan et al., 1975a,b).

Materials and Methods

Materials. Avian PP was prepared by the method described by Kimmel et al. (1975). All other chemicals were of reagent grade or the equivalent.

Large-Zone Analytical Gel Chromatography. Molecular sieve chromatography was performed in buffers of 0.1 ionic strength as described earlier (Noelken et al., 1980) with the following modifications for large-zone experiments. (1) The jacketed 0.9 × 150 cm column was maintained at the desired temperature ± 0.5 °C. (2) To ensure the formation of a constant plateau concentration equal to the initial sample concentration, we applied sample volumes of 12–15 mL to the column with the use of a Marriotte flask. Samples were degassed and the temperature was equilibrated before application. (3) The relative elution protein concentration was continuously measured with a refractive index monitor equipped with a chart recorder. Absolute protein concentration was determined spectrophotometrically at 276 nm with a Zeiss single-beam spectrophotometer using quartz cuvettes with 1.0-cm light paths. The extinction coefficient for avian PP used for this study was 1.45 OD_{276}/(mg mL) in 0.1 M acetic acid (Kimmel et al., 1975). This value was found to be essentially unchanged in the solvents used in this study. Protein concentrations less than 20 μg/mL were determined by radioimmunoassay as described by Langslow et al. (1973). (4) The weight-average partition coefficient $\bar{\sigma}_w$ for avian PP at the concentration corresponding to the plateau region of the elution curve boundary was determined by using the equation (Ackers & Thompson, 1965)

$$\bar{\sigma}_w = \frac{\bar{V} - V_0}{V_i} \quad (1)$$

The void volume V_0 was determined as the elution volume of bovine serum albumin, while the internal volume V_i was determined by the elution volume of NaCl, monitored by conductivity, subtracted from V_0.

The centroid elution volume $\bar{V}$, corresponding to the leading edge of the elution boundary and representing the first moment of the leading edge (Longsworth, 1943), was determined by planimetry by using the relation

$$\bar{V} = \frac{1}{C_0}\int_0^{C_0} V\,dC \quad (2)$$

where C_0 is the plateau concentration, V is the volume, and C is the concentration within the boundary (Ackers & Thompson, 1965).

Results

Elution Curve Profiles. Qualitative information about the association can be derived from an examination of the shapes

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[1] Abbreviation used: PP, pancreatic polypeptide.

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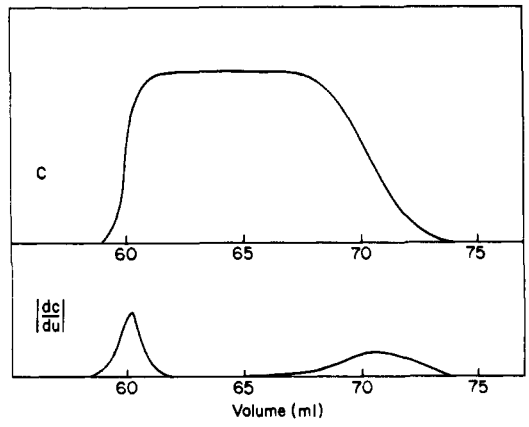

FIGURE 1: Typical elution profile and first-derivative curve obtained in large-zone gel chromatography of avian PP (0.144 mg/mL) on a column of Bio-Rad P-30 in Tris-HCl buffer, pH 8.0. Temperature was 30 °C.

of the leading and trailing boundaries of the large-zone elution curve profile. Figure 1 shows a representative elution profile obtained for avian PP. In order to facilitate interpretation of the hormone's elution pattern in relation to the mass transport theories presented by Gilbert and others (Gilbert, 1955; Winzor & Scheraga, 1963; Ackers & Thompson, 1965), we also present a first-derivative curve of the elution pattern with respect to volume.

As can be seen from Figure 1, the elution profile features a sharp leading edge and a diffuse trailing edge. According to the postulates presented by Winzor & Scheraga (1963), these features indicate a concentration-dependent, rapid equilibrium between the avian PP monomer and a higher polymeric species.

Stoichiometry. Ackers & Thompson (1965) have shown from their mass transport equations that the stoichiometry of an associating system can be determined by an analysis of the trailing edge of the elution profile. The equations predict a unimodal first-derivative curve, with a single maximum, for dimerizing systems and a bimodal gradient curve, with a single minimum, for higher order polymerization. It is therefore possible to distinguish between a dimerization scheme and a higher order associating system.

An examination of Figure 1 shows that the first-derivative curve corresponding to the trailing edge of the elution profile for avian PP is unimodal, with a single maximum at 70.6 mL. This result gives strong evidence for the hormone's dimerization and supports the conclusions reached earlier (Noelken et al., 1980).

Concentration Dependence. Additional evidence for the concentration dependence of avian PP dimerization was provided when weight-average partition coefficients, $\bar{\sigma}_w$, were determined as a function of hormone concentration at various pH values. It can be seen in Figure 2 that $\bar{\sigma}_w$ decreases with increasing concentration, thereby indicating a shift to increased association. The increase in the degree of association with increasing pH from pH 4.0 to pH 8.0 can also be observed in Figure 2.

Calculation of Dimerization Equilibrium Constants. Association constants for avian PP dimerization, K_D, were calculated according to the relationship (Ackers & Thompson, 1965)

$$K_D = \frac{1 - \alpha}{n\alpha^n C_0^{n-1}} \tag{3}$$

where C_0 is the total protein plateau concentration in moles of monomer per moles of solvent and n is the stoichiometry

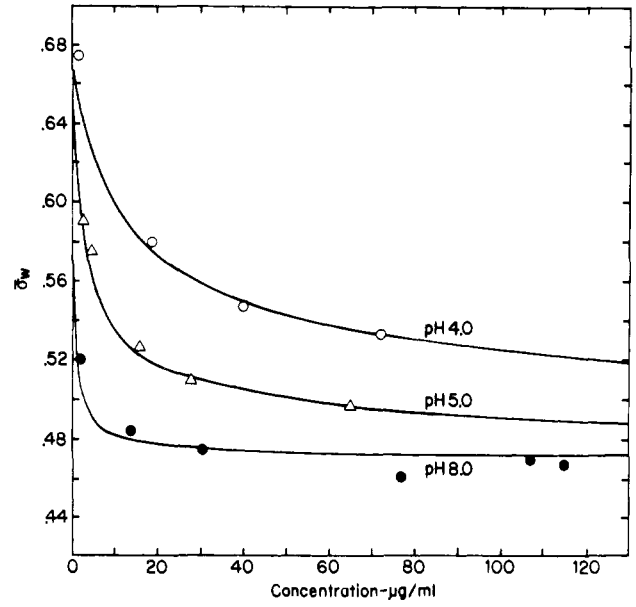

FIGURE 2: Weight-average partition coefficient, $\bar{\sigma}_w$, of avian PP as a function of concentration. Partition coefficients were calculated from large-zone gel chromatography experiments using eq 1 and 2.

of the association ($n = 2$). The weight fraction of monomer, α, is determined by (Ackers & Thompson, 1965)

$$\alpha = \frac{\bar{\sigma}_w - \sigma_D}{\sigma_M - \sigma_D} \tag{4}$$

where σ_D is the partition coefficient for the avian PP dimer and σ_M is the partition coefficient for the monomer.

σ_M and σ_D must be carefully determined in order to ensure accuracy in the calculation of K_D. In the present study, partition coefficients were determined under conditions that would shift the equilibrium to maximize either the monomer or dimer concentration. Therefore, the pH dependence of the peptide dimerization was exploited, along with its concentration dependence. For the determination of σ_M, very low concentrations (<1.0 μg/mL) of the hormone were used at pH 4.0. Conversely, high concentrations (>100 μg/mL) of avian PP were used at pH 8.0 to determine σ_D. The partition coefficients determined in this way were 0.650 ± 0.002 for σ_M and 0.467 ± 0.003 for σ_D.

Support for the accuracy of the above values comes from an examination of Figure 2. An estimate of σ_M was obtained by extrapolation of these curves to infinite dilution. As can be seen from the figure, the σ_M value determined above is in excellent agreement with such an extrapolation. Similarly, an analysis of the pH 8.0 curve indicates that the partition coefficient does not change significantly at concentrations above 50 μg/mL and the minimum value is in excellent agreement with that determined above.

Effect of pH on K_D. Table I presents the values of K_D at various pH values as well as the data from which these constants were derived.

We interpreted the pH dependence of the association on the basis of the following set of reactions:

$$nA \rightleftharpoons A_n$$

$$A \rightleftharpoons B + qH^+$$

$$nB \rightleftharpoons B_n$$

where A and B are monomers, each of which can associate to form the products A_n and B_n, respectively, and n has the value 2 for avian PP association. Species B is formed by the dissociation of q protons from A; we considered two possi-

Table I: pH Dependence of Avian PP Dimerization Constant[a]

pH	protein concn (M)	$\bar{\sigma}_w$	α	K_D (M^{-1})	mean K_D (M^{-1})
4.0	1.67×10^{-5}	0.534	0.322	1.96×10^5	2.01×10^5
	9.19×10^{-6}	0.550	0.399	2.06×10^5	
5.0	1.51×10^{-5}	0.497	0.144	1.36×10^6	1.42×10^6
	6.34×10^{-6}	0.510	0.207	1.47×10^6	
6.0	1.24×10^{-5}	0.475	0.038	2.69×10^7	2.47×10^7
	5.24×10^{-6}	0.480	0.063	2.25×10^7	
8.0	3.07×10^{-6}	0.481	0.067	3.35×10^7	3.48×10^7
	7.08×10^{-6}	0.476	0.043	3.61×10^7	
8.6	3.25×10^{-6}	0.481	0.066	3.31×10^7	3.39×10^7
	5.07×10^{-6}	0.478	0.052	3.46×10^7	

[a] K_D values were determined in duplicate as described in the text and were calculated by using eq 3.

bilities. (1) The first is a proton dissociation process in which the q sites are independent and equal and B aggregates with an association constant K_0. All other monomer species, i.e., those which have lost less than q protons (collectively labeled A), have an association constant of K_H. (2) The second is a fully cooperative proton dissociation process involving only two monomeric species A and B, which have association constants of K_0 and K_H, respectively.

In either treatment it is assumed that A and B are identical with respect to gel filtration behavior and that A_n and B_n likewise have identical partition coefficients. This assumption is warranted by the observation that there is no significant conformation change in avian PP on going from pH 4 to pH 8 (Noelken et al., 1980). K_n, the observed association constant, is related to the various polypeptide species by

$$K_n = \frac{[A_n] + [B_n]}{([A] + [B])^n} \tag{5}$$

In the situation where protons are released independently from species A and have equal dissociation constants, K_a, it can be shown that [B] is related to the total monomer concentration by the relation

$$\frac{[B]}{[A] + [B]} = \frac{1}{(1 + [H]/K_a)^q} \tag{6}$$

where [H] is the hydrogen ion concentration. Recognizing that

$$K_H = [A_n]/[A]^n \tag{7}$$

and

$$K_0 = [B_n]/[B]^n \tag{8}$$

appropriate substitutions involving eq 6–8 can be made in eq 5 to obtain

$$K_n = \frac{K_0 + K_H[(1 + [H]K_a)^q - 1]^n}{(1 + [H]/K_a)^{nq}} \tag{9}$$

In the mechanism involving cooperative proton release, A is related to B by

$$[A] = [B][H]^q/K_a \tag{10}$$

and eq 5 can be modified by use of eq 7, 8, and 10 to yield

$$K_n = \frac{K_0 + K_H([H]^q/K_a)^n}{(1 + [H]^q/K_a)^n} \tag{11}$$

Plots of the logarithm of K_D vs. pH are shown in Figure 3. The points represent experimentally determined values, and the three curves were calculated by using the independent and

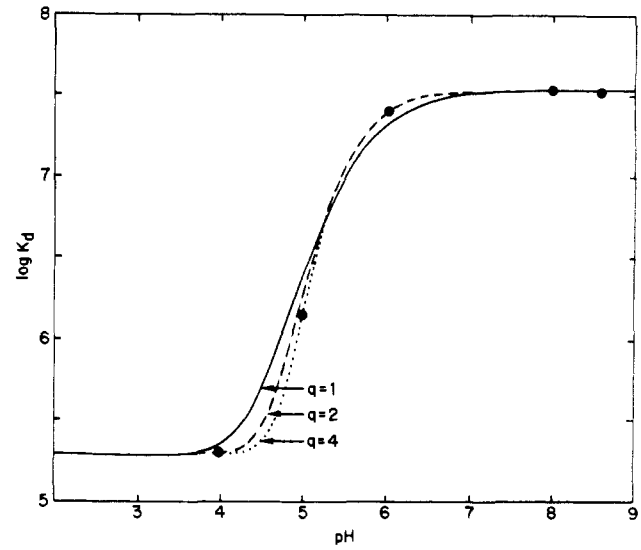

FIGURE 3: Dimerization constant, K_D, of avian PP as a function of pH. Experimental points were derived from data presented in Table I while curves were calculated by using eq 9 for q = 1, 2, and 4. The curve for q = 6 is essentially the same as that for q = 4.

Table II: Temperature Dependence of Avian PP Dimerization Constants[a]

T (K)	protein concn (X_a)	$\bar{\sigma}_w$	α	K_D (M^{-1})	mean K_D (M^{-1})
281	1.17×10^{-7}	0.657	0.913	4.46×10^5	5.81×10^5
	1.98×10^{-7}	0.636	0.813	7.15×10^5	
286	1.58×10^{-7}	0.597	0.625	3.04×10^6	2.92×10^6
	1.87×10^{-7}	0.594	0.611	2.80×10^6	
291	1.14×10^{-7}	0.544	0.370	2.02×10^7	1.68×10^7
	1.26×10^{-7}	0.554	0.418	1.34×10^7	
296	1.70×10^{-7}	0.536	0.332	1.79×10^7	1.81×10^7
	1.80×10^{-7}	0.534	0.322	1.82×10^7	
301	1.82×10^{-7}	0.523	0.269	2.78×10^7	3.24×10^7
	2.65×10^{-7}	0.509	0.202	3.70×10^7	
306	2.72×10^{-7}	0.497	0.144	7.60×10^7	7.85×10^7
	1.14×10^{-7}	0.510	0.207	8.10×10^7	
311	2.68×10^{-7}	0.472	0.024	3.16×10^9	3.36×10^9
	2.38×10^{-7}	0.472	0.024	3.55×10^9	
316	7.33×10^{-8}	0.477	0.048	2.81×10^9	2.56×10^9
	7.35×10^{-8}	0.478	0.053	2.30×10^9	

[a] K_D values were determined at pH 5.0 at various temperatures as described in the text and were calculated by using eq 3.

equal sites model (eq 9), with values for q of 1, 2, 4, and 6. For each curve, K_0, the association constant at pH 8.0, has the value 3.48×10^7 M^{-1} and K_H, the association constant at pH 4.0, is 2.01×10^5 M^{-1}. The pK_a values for q = 1, 2, 4, and 6 were found to be 5.45, 5.03, 4.71, and 4.50, respectively. Curve fitting using the cooperative model for proton release did not result in a satisfactory fit to the data.

Temperature Dependence and Thermodynamic Parameters. In order to calculate the thermodynamic parameters behind avian PP dimerization, we determined association constants as a function of temperature at pH 5.0. The K_D values were measured at pH 5.0 so that $\bar{\sigma}_w$ values would be approximately equidistant from σ_M and σ_D, thus ensuring precise determination of α and K_D throughout the temperature range. Table II presents the experimental data used to calculate K_D values from 5 to 40 °C. As can be seen from the table, dimerization constants are calculated by using hormone concentrations expressed in mole fraction units. This ensures that thermodynamic parameters derived from K_D will be unitary values, including no contribution from cratic entropy terms (Gurney,

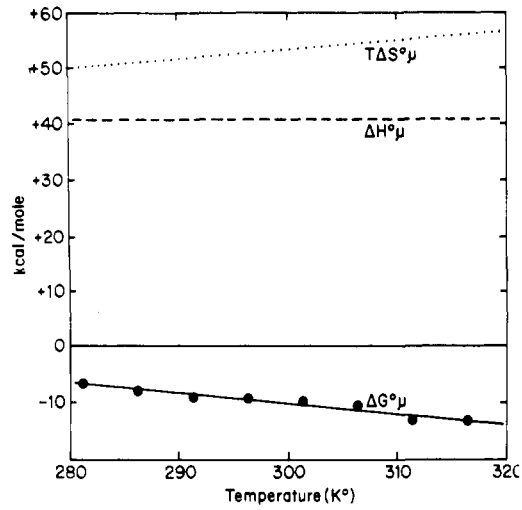

FIGURE 4: Unitary thermodynamic parameters for avian PP self-association at pH 5.0. Experimental points for ΔG°_μ were derived from the data in Table II by using eq 12. Curves for ΔG°_μ, $T\Delta S^\circ_\mu$, and ΔH°_μ were calculated by using eq 13, 14, and 15.

1953; Kauzmann, 1959; Tanford, 1973). Unitary free energy changes ΔG°_μ were calculated by using the relation

$$\Delta G^\circ_\mu = -RT \ln K_D \quad (12)$$

where the gas constant R is expressed in kcal/(deg mol) and K_D is expressed in unitary (reciprocal mole fraction) units.

Free energy changes were fit to an empirical polynomial expansion in temperature by using a nonlinear recurrence regression algorithm incorporating orthogonal polynomials (Pizer, 1975). The remaining thermodynamic parameters were calculated from the appropriate derivative relations (Dickerson, 1969):

$$\Delta G^\circ_\mu = \sum_{k=0}^{M} C(k) T^k \quad (13)$$

$$\Delta S^\circ_\mu = \frac{-\partial(\Delta G^\circ)}{\partial T} = -\sum_{k=0}^{M} kC(k) T^{k-1} \quad (14)$$

$$\Delta H^\circ_\mu = \frac{\partial(\Delta G^\circ)}{\partial(1/T)} = \sum_{k=0}^{M} (1-k) C(k) T^k \quad (15)$$

The order of polynomial M was determined by calculating the variance estimator σ^2 for increasing M. The order of polynomial which corresponded to the smallest significant σ^2 value was chosen as M (Pizer, 1975). The coefficients of the polynomial expansion $C(k)$ were then calculated by using the recurrence algorithm.

This nonlinear expansion was used instead of the classical van't Hoff relationship since it was not known at the time whether or not a change in heat capacity occurred with increasing temperature. If such a change had occurred, the van't Hoff relationship would not have given valid results (van Holde, 1971).

Figure 4 has a plot of ΔG°_μ vs. temperature for avian PP dimerization, derived from the data of Table II. The points represent experimentally derived values while the curve was calculated from eq 13. Using the polynomial regression algorithm, we found that the values of M, $C(0)$, and $C(1)$ which gave the best fit to the data were 1, 41.611, and −0.178, respectively.

Figure 4 also shows application of the polynomial expansion to calculate $T\Delta S^\circ_\mu$ and ΔH°_μ for avian PP dimerization as a function of temperature by using eq 14 and 15. From the figure, ΔH°_μ was found to be +41.6 ± 6.3 kcal/mol of monomer, while ΔS°_μ was found to be +178.2 ± 26.8 cal/(deg mol of monomer). The uncertainties in these values reflect the degree of uncertainty in the determination of $\bar{\sigma}_w$ and hormone concentration values necessary for calculation of K_D. The total contribution from these errors was estimated to result in an uncertainty of no more than 15% for all thermodynamic parameters derived from the experimentally determined K_D values.

	1										6										12
BOVINE:	Ala	-	Pro	-	Leu	-	Glu	-	Pro	-	Glu	-	Tyr	-	Pro	-	Gly	-	Asp	-	Asn	-	Ala
CANINE:	Ala	-	Pro	-	Leu	-	Glu	-	Pro	-	Val	-	Try	-	Pro	-	Gly	-	Asp	-	Asp	-	Ala
CHICKEN:	Gly	-	Pro	-	Ser	-	Gln	-	Pro	-	Thr	-	Tyr	-	Pro	-	Gly	-	Asp	-	Asp	-	Ala

	13										18										24
BOVINE:	Thr	-	Pro	-	Glu	-	Gln	-	Met	-	Ala	-	Gln	-	Tyr	-	Ala	-	Ala	-	Glu	-	Leu
CANINE:	Thr	-	Pro	-	Glu	-	Gln	-	Met	-	Ala	-	Gln	-	Tyr	-	Ala	-	Ala	-	Glu	-	Leu
CHICKEN:	Pro	-	Val	-	Glu	-	Asp	-	Leu	-	Ile	-	Arg	-	Phe	-	Tyr	-	Asp	-	Asn	-	Leu

	25										30										36		
BOVINE:	Arg	-	Arg	-	Tyr	-	Ile	-	Asn	-	Met	-	Leu	-	Thr	-	Arg	-	Pro	-	Arg	-	Tyr	-	NH_2
CANINE:	Arg	-	Arg	-	Tyr	-	Ile	-	Asn	-	Met	-	Leu	-	Thr	-	Arg	-	Pro	-	Arg	-	Tyr	-	NH_2
CHICKEN:	Gln	-	Gln	-	Tyr	-	Leu	-	Asn	-	Val	-	Val	-	Thr	-	Arg	-	His	-	Arg	-	Tyr	-	NH_2

FIGURE 5: Amino acid sequence of avian PP (Kimmel et al., 1975) and bovine PP and canine PP (Chance et al., 1979).

Discussion

An important objective of this study was to test the tentative conclusion reached earlier (Noelken et al., 1980), namely, that the self-association of avian PP was a concentration- and pH-dependent dimerization. In the present study, integral boundary analysis of avian PP elution profiles (Figure 1) has shown that avian PP does indeed undergo a rapidly equilibrating dimerization, while the results presented in Figures 2 and 3 clearly demonstrate the equilibrium's concentration and pH dependence. With the existence of hormone dimerization demonstrated, a closer, more quantitative examination of the driving forces involved was possible. An examination of Figure 4 clearly shows that avian PP dimerization becomes more favorable with increasing temperature. In addition, the results presented in Figure 4 indicate that this association is endothermic and entropically driven, with ΔH°_μ = +41.6 ± 6.3 kcal/mol and ΔS°_μ = +178.2 ± 26.8 eu.

The usual interpretation of entropically driven protein–protein association is that hydrophobic interactions are involved, where the entropy increase due to the transfer of water from hydrophobic interfaces into the bulk solvent is the driving force for association. To determine whether or not the observed change in entropy of avian PP dimerization can be reasonably accounted for by hydrophobic interaction, we used data from previous X-ray (Wood et al., 1977), circular dichroism, and conformation prediction (Noelken et al., 1980) studies. These studies indicate that the avian PP molecule has significant helical structure. When residues 13–32 are plotted as a Schiffer & Edmundson (1967) helical wheel, a very well-defined hydrophobic face containing nine residues is apparent [see Figure 3, Noelken et al. (1980)]. In the remainder of the molecule, hydrophobic side chains are also found on the three proline residues and histidine-34, which is neutral at pH 8.0 (Figure 5). Therefore, there are a total of 13 hydrophobic residues per avian PP monomer that may possibly be properly oriented for hydrophobic interactions leading to dimerization.

The free energy of transfer of a number of hydrophobic side chains from organic solvents to water has been determined (Brandts, 1964; Fugita et al., 1964). Since it has been found that the contributions from these side chains are additive

(Fugita et al., 1964), the total contribution of the above hydrophobic side chains to the possible hydrophobically driven association of avian PP can be estimated.

Although the free energy of transfer values for all of the side chains that may possibly be involved in avian PP self-association have not been determined, an average value per amino acid residue can be approximated. This approach is valid as long as the residues involved do not differ greatly in accessible surface area (Hermann, 1972). Incorporating data from the available literature (Brandts, 1964; Fugita et al., 1964; Hansch & Coats, 1970; Leo et al., 1971; Edelhoch & Osborne, 1976), it was found that, on the average, the hydrophobic side chains of avian PP can contribute $\sim 11.6 \pm 2.5$ eu/residue to hydrophobic interactions.

The total entropy contribution that could be expected for such an interaction is therefore 13×11.6, or 150.8 ± 32.5 eu. Since this is within experimental error of the observed entropy change for the hormone dimerization, hydrophobic interactions could indeed account for the observed entropy change accompanying peptide dimerization. Admittedly, this kind of calculation is crude. However, a more rigorous and refined estimate of the entropy change due to hydrophobic interaction in self-associating systems is a difficult task, due to the lack of a completely satisfactory model. There are significant differences between the hydrophobic interactions involved in solvent transfer experiments and polymerizing systems. The transfer of solute from the aqueous to the organic phase in solvent transfer experiments involves the formation of a "cavity" for the solute in the organic phase, along with the closing of the cavity left in the aqueous phase. This contrasts with associating peptides, where hydrophobic interactions between subunits involve preformed hydrophobic faces or cavities (Fersht, 1977), which are characterized by significantly stronger hydrophobic interactions. For example, the hydrophobic binding pocket of chymotrypsin appears to be twice as hydrophobic as would be predicted by solvent transfer data (Dorovskaya et al., 1972; Fastrez & Fersht, 1973). A similar situation might exist for peptide self-associating systems involving hydrophobic faces, including avian PP.

In addition to the observed favorable change in entropy, the polymerization of avian PP also appears to be accompanied by a rather large and unfavorable enthalpy change. Since circular dichroism studies indicated that no significant conformational change occurs during peptide self-association (Noelken et al., 1980), an alternative interpretation must be found for this large change. If one assumes that avian PP dimerization is accomplished through interaction between the indicated hydrophobic faces of two peptide molecules, there is the possibility that peptide–water hydrogen bonds that might be formed in the monomer by certain residues located at the hydrophobic face may be broken during dimerization due to the entropically driven release of water from the hydrophobic interface. The observed enthalpy change could be accounted for if the energy from these broken bonds is not recovered by the formation of new intramolecular hydrogen bonds (about 5 kcal/mol; Fersht, 1977).

Another important observation concerning avian PP dimerization is its pH dependence. As can be seen in Figure 3, dimerization is enhanced with increasing pH between pH 4.0 and pH 8.0. We found that the experimental results could be interpreted in terms of a model in which the release of protons from equal and independent sites on one species of monomer converted it into a second species which had a greater tendency to dimerize. Although the exact number of proton-binding sites could not be obtained unambiguously from such an analysis, inspection of the sequence (Figure 5) limits the maximum number of such sites to six. The calculated pK_a of the group, or groups, involved must be in the range 4.5–5.5, since the value is not very sensitive to the number of groups hypothesized. An exact interpretation of the observed pK_a is to be avoided, since the dimerization constants at pH 8.0 and above were determined from data in which less than 7% of the avian PP molecules were monomers, in which case a small amount of inhomogeneity or impurity of monomer size could result in significant errors in K_0 and, hence, in pK_a. The net effect of this would be to make the observed pK_a too low.

Inspection of the sequence of avian PP (Figure 5) indicates that there are several titratable groups with a pK_a of ~ 5. Histidine-34 is flanked by two arginine residues, and this could cause its pK_a to be lower than the typical value of 6.3 (Tanford, 1962). Also, there are five carboxyl groups, on residues 10, 11, 15, 16, and 22, which would have pK_a values of ~ 4.7 (Tanford, 1962) if they were in a normal environment. The propinquity of residues 10 and 11, and 15 and 16, and the possibility that these four residues are in close proximity in the three-dimensional structure would cause one or more of them to have pK_a values greater than 5, at the upper end of the range that we observed.

A comparison of the sequence of avian PP with that of bovine PP and canine PP reveals that the ionizable groups which they have in common are Asp-10, Asp-11, Glu-15, Arg-33, and Arg-35 (Figure 5). Since the three hormones exhibit a similar pH-dependent association (Noelken et al., 1980), it seems quite likely that carboxylate–guanidinium salt links are involved in the dimerization. In the case of avian PP, the increased hydrophobic character of the His-34 imidazole at pH 8 and its protonation at lower pH could also affect the association behavior. It is significant that a proline residue, with a hydrophobic side chain, occupies position 34 in the mammalian peptides.

It has been suggested that the hydrophobic residues involved in the self-association of glucagon (Sasaki et al., 1975) and insulin (Pullen et al., 1976) are also involved in the association with their respective membrane receptor. Thus, a three-way equilibrium between peptide monomer, polymer, and monomer–receptor is thought to exist.

The concentration of avian PP in chicken plasma is ~ 1.2 nM (Kimmel et al., 1978), and at physiological conditions (body temperature = 41.5 °C, pH ~ 8.0; Sturkie, 1976), a significant concentration of dimer (50–90%) would be expected. Since it is likely that avian PP interacts with a specific receptor in the body, it is quite possible that a similar equilibrium might be operative. It is noteworthy that avian PP also has a hydrophobic face involved in self-association.

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Polyamine–Polyphosphate Complexes as Enzyme Inhibitors†

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ABSTRACT: By use of the anion-exchange resin method, the apparent complex formation constants of Mg^{2+} and three polyamines with 5-phosphoribosyl 1-pyrophosphate (PRibPP) and 2,3-diphosphoglyceric acid (2,3-DPG) have been obtained. Binding of polyamines to PRibPP seems to have protected the compound from nonenzymatic hydrolysis. Analysis of the polyamine inhibition data has shown that both PRibPP– and 2,3-DPG–polyamine complexes themselves are inhibitors for enzymes. The PRibPP–spermine complex is a competitive inhibitor for hypoxanthine phosphoribosyltransferase (HPRTase, EC 2.4.2.8), and the 2,3-DPG–spermine complex is an uncompetitive inhibitor for 2,3-DPG phosphatase. Evaluation of the interaction of polyamines with PRibPP suggests that the effects of polyamines on PRibPP metabolism are complex. The compounds interact with PRibPP to form stable competitive inhibitors of many enzymes that utilize Mg^{2+}–PRibPP. The effectiveness of polyamine inhibition is a function of the Mg^{2+} concentration. In the absence of PRibPP, polyamine binding decreases the heat stability of adenine phosphoribosyltransferase (APRTase, EC 2.4.2.7) but protects PRibPP synthetase (EC 2.7.6.1) against thermal inactivation and changes the gel filtration pattern of the latter enzyme. The activity of the synthetase can be inhibited, activated, or not affected by polyamines depending upon assay conditions. Polyamines also interact with purine nucleotides and consequently change the apparent end product inhibition produced by these compounds on PRibPP synthetase and glutamine PRibPP amidotransferase (EC 2.4.2.14). ADP or 2,3-DPG can partially reverse the spermine inhibition of PRibPP synthetase. Spermine on the other hand affects feedback inhibition of amidotransferase. The polyamine inhibition constants for some of the PRibPP-utilizing enzymes have been calculated.

The polyamines are widely distributed in biological systems. Due to their polycationic nature, they not only bind to the highly negative nucleic acids but also form stable complexes with a number of polyphosphate compounds and affect many enzyme reactions (Nakai & Glinsmann, 1977a; Lovgren et al., 1978; Usui et al., 1978; Liang et al., 1979; Queigley et al., 1978; Killilea et al., 1978). However, no detailed analysis of the mechanism of polyamine effects on substrate binding has been performed. A general nonspecific substrate deprivation mechanism was assumed to be responsible for the observed inhibition of many enzyme reactions. Therefore, the biological significance of substrate–polyamine complex binding to the enzyme has not yet been established.

When an inhibitor (polyamine) binds with a substrate (polyphosphate), the inhibition observed could be due to either of the following: (1) the inhibitor combines with the substrate and decreases its effective concentration or (2) the inhibitor–substrate complex is itself an inhibitor of the enzyme.

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