Biochemistry 1993, 32, 11638–11645

The Allosteric Transition of the Insulin Hexamer Is Modulated by Homotropic and Heterotropic Interactions[†]

Wonjae E. Choi,[‡] Mark L. Brader,[‡] Valentin Aguilar,[‡] Niels C. Kaarsholm,[§] and Michael F. Dunn*,[‡]

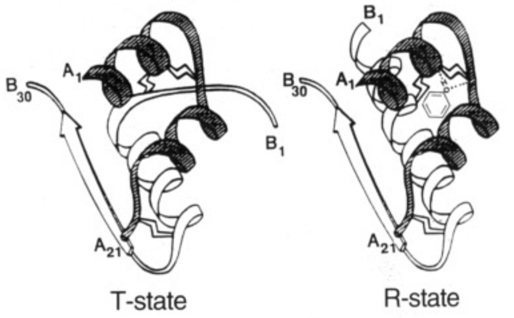
Department of Biochemistry—015, University of California, Riverside, Riverside, California 92521-0129, and Novo Research Institute, DK 2880, Bagsvaerd, Denmark

Received March 26, 1993; Revised Manuscript Received August 18, 1993[®]

ABSTRACT: The allosteric behavior of the Co(II)-substituted insulin hexamer has been investigated using electronic spectroscopy to study the binding of different phenolic analogues and singly charged anions to effector sites on the protein. This work presents the first detailed, quantitative analysis of the ligand-induced T- to R-state allosteric transition of the insulin hexamer. Recent studies have established that there are two ligand binding processes which stabilize the R-state conformation of the Co(II)-substituted hexamer: the binding of cyclic organic molecules to the six protein pockets present in the Zn(II)–R_6 insulin hexamer [Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Reynolds, C. D., Smith, G. D., Sparks, C., & Swensen, D. (1989) *Nature 338*, 594–596] and the coordination of singly charged anions to the His(B10) metal sites [Brader, M. L., Kaarsholm, N. C., Lee, W. K., & Dunn, M. F. (1991) *Biochemistry 30*, 6636–6645]. The R_6 insulin hexamer is stabilized by heterotropic interactions between the hydrophobic protein pockets and the coordination sites of the His(B10)-bound metal ions. The binding studies with 4-hydroxybenzamide, *m*-cresol, resorcinol, and phenol presented herein show that, in the absence of inorganic anions, the 4-hydroxybenzamide-induced transition, with a Hill number of 2.8, is the most cooperative, followed by *m*-cresol, phenol, and resorcinol with Hill numbers of 1.8, 1.4, and 1.2, respectively. The relative effectiveness of these ligands in shifting the allosteric equilibrium in favor of the Co(II)–R_6 hexamer was found to be resorcinol > phenol > 4-hydroxybenzamide > *m*-cresol. In the presence of inorganic anions, the binding isotherms become less sigmoidal, the Hill numbers approach 1, and the apparent affinities increase. Hence, these monodentate anions behave as positive allosteric effectors by coordinating to the His(B10)-bound Co(II) ions. The Co(II)-substituted insulin hexamer is further stabilized by divalent metals such as Ca^{2+} and Cd^{2+}, which are known to bind to the Glu(B13) cage located in the center of the insulin hexamer [Hill, C. P., Dauter, Z., Dodson, E. J., Dodson, G. G., & Dunn, M. F. (1991) *Biochemistry 30*, 917–924]. Therefore, the R-state insulin hexamer is stabilized by a variety of heterotropic and homotropic interactions involving the coordination of singly charged anions to the His(B10)-bound metal ions, hydrogen bonding, and van der Waals interactions between the cyclic organic molecules and the protein pockets.

Allosteric conformational transitions provide one mechanism by which proteins transmit biochemical information. However, there are few examples of allosteric proteins for which high-resolution crystal structures of both the T- and R-states and the associated effector complexes have been determined (Perutz, 1989). The insulin hexamer is a recent addition to this category. Single-crystal X-ray diffraction studies have provided high-resolution structures of three rhombohedral forms and a monoclinic form of the insulin hexamer (Schlichtkrull, 1958; Blundell et al., 1972; Peking Insulin Structure Research Group, 1974; Bentley et al., 1976; Dodson et al., 1979; Sakabe et al., 1981; Smith et al., 1984; Smith & Dodson, 1992a,b; Baker et al., 1988; Derewenda et al., 1989, 1991). These structures have been designated as the T_6 (formerly 2Zn), T_3R_3 (formerly 4Zn), and R_6 (formerly 2Zn phenol-induced) species, respectively, by Kaarsholm et al. (1989), who proposed the T_6, T_3R_3, and R_6 nomenclature[1] in recognition that these are interconvertible allosteric states which possess different spectroscopic and chemical properties and different ligand binding affinities. The structure of the R_6 hexamer has been refined to 1.8-Å resolution (Derewenda et al., 1989), whereas the T_6 and T_3R_3 structures both have been refined to 1.5-Å resolution. The T_6, T_3R_3, and R_6 hexamers all possess torus-shaped structures that bind two Zn^{2+} ions at the His(B10) chelate sites (Chart I).

Chart I: Ribbon Diagrams Showing the Structural Difference between the T Conformation and the Phenol-Bound R Conformation of the Insulin Monomer[a]



[a] Redrawn with permission from Brader et al. (1992).

Phenol induces a large change in the conformation of the insulin hexamer, both in the crystalline state (Smith et al.,

---

[†] Supported by NIH Grant 5 R01-DK42124 and a grant from the Novo Research Institute.

* To whom correspondence should be addressed.

[‡] UC Riverside.

[§] Novo Research Institute.

[®] Abstract published in *Advance ACS Abstracts*, October 1, 1993.

[1] Abbreviations: T and R designate the conformations of an insulin subunit in the two-zinc hexamer and the phenol-induced hexamer, respectively (Kaarsholm et al., 1989). T_6, T_3R_3, and R_6 are the three crystallographically defined allosteric forms of the insulin hexamer.

0006-2960/93/0432-11638$04.00/0

1984; Baker et al., 1988; Derewenda et al., 1989, 1991; Smith & Dodson, 1992a,b) and in solution (Roy et al., 1989; Thomas & Wollmer, 1989; Kaarsholm et al., 1989; Brader et al., 1990, 1991, 1992a,b; Kruger et al., 1990; Brader & Dunn, 1990, 1991). The X-ray crystal structures of the Zn(II) hexamers show that, in the presence of phenol, residues B1–B8 of each monomer undergo conversion from an extended chain to a helix (Smith et al., 1984; Baker et al., 1988; Derewenda et al., 1989, 1991; Smith & Dodson, 1992a,b), causing some residues to move as much as 30 Å. This alteration of the hexamer topology creates six essentially equivalent phenol binding sites (one per monomer). The binding of each phenol molecule in the monoclinic R_6 structure involves hydrogen bonds between the hydroxyl group of the phenol and the carbonyl oxygen of Cys(A6) and the amide nitrogen of Cys(A11) and favorable van der Waals contacts between the phenol ring and several side chain residues (Derewenda et al., 1989). These hydrophobic pockets are occluded in the T_6 conformation by the Leu(B6) side chain. In the Zn(II)–T_6 hexamer, the two Zn^{2+} ions are coordinated in an octahedral arrangement by the three His(B10) imidazole ring nitrogens and by three water molecules (Baker et al., 1988; Hill et al., 1991). In the monoclinic Zn(II)–R_6 hexamer, the two Zn^{2+} ions are coordinated in a tetrahedral arrangement by the same His(B10) nitrogens and a Cl^- ion (Derewenda et al., 1989). The electron density maps of the rhombohedral Zn(II)–R_6 structure (Smith & Dodson, 1992a) indicate that a phenolate ion is coordinated to one of the two Zn^{2+} ions. In solution, phenol binds both to the protein pockets created by the Co(II)–T_6 to Co(II)–R_6 transition and (as phenolate) to the fourth (exchangeable) ligand position of each metal site (Roy et al., 1989; Brader et al., 1990, 1991; Brader & Dunn, 1991).

The recognition that the insulin hexamer is an allosteric protein (Kaarsholm et al., 1989), combined with the determination of the high-resolution crystal structures for the three conformational states (Baker et al., 1988; Smith et al., 1984; Derewenda et al., 1991; Smith & Dodson 1992a,b), make the $T_6 \rightleftharpoons R_6$ transition an attractive system for studying the structural basis and mechanism of an allosteric transition. Prior studies of metal-substituted insulin hexamers (Roy et al., 1989; Brader et al., 1990, 1991, 1992a,b; Brader & Dunn, 1990, 1991; Gross & Dunn, 1992) presented largely qualitative evidence demonstrating that the T- to R-state transition is an allosteric process. The work presented herein shows that the mediation of the T- to R-state transition in the Co(II)-substituted insulin hexamer by the binding of phenolic compounds to the protein pockets involves apparent positive cooperative homotropic interactions among the six phenolic protein pockets. Furthermore, monodentate anions such as Cl^- and SCN^- are positive heterotropic effectors of phenol binding. These strong heterotropic allosteric interactions involve the fourth coordination position of the metal center and the phenolic protein pockets. The present study presents a quantitative analysis of the ligand-binding interactions which characterize the allosteric behavior of the Co(II)-substituted insulin hexamer.

MATERIALS AND METHODS

Materials. The chemicals employed in these studies were reagent grade or better and were used without further purification. Metal-free human insulin was a gift supplied by Novo-Nordisk (Denmark). Phenol was purchased from J. T. Baker Inc. Resorcinol and Trizma base were purchased from Sigma. Potassium thiocyanate and perchloric acid were purchased from Mallinckrodt. Sodium chloride was purchased from Fisher Scientific. Cobalt perchlorate was purchased from Alfa Products. 4-Hydroxybenzamide and *m*-cresol were purchased from Aldrich.

Table I: Ligand Dependence of L_{sat}

phenolic pocket ligands[a] (at saturation)	Co(II)-adduct[a] (at saturation)	apparent ratio of octahedral to tetrahedral centers[b]
none	none	>100 or 1000
none	SCN	1.8
phenol	none	0.4
phenol	Cl	0.12
phenol	SCN	0.05
resorcinol	none	0.11
resorcinol	Cl	0.05
resorcinol	SCN	<0.01
m-cresol	none	6
m-cresol	Cl	0.6
m-cresol	SCN	0.2
4-hydroxybenzamide	none	0.35
4-hydroxybenzamide	Cl	
4-hydroxybenzamide	SCN	

[a] Experiments were carried out in Tris-perchlorate buffer (50 mM) at pH 8.0 and 25 °C. The entry "none" refers to experiments where no ligand [for the phenolic pocket and/or the fourth coordination of the Co(II) center] has been added in addition to the buffer and lyate species present. Where a ligand has been added, it is present in an amount sufficient to saturate any accompanying spectrals changes in the d → d envelope of the Co(II) chromophore. [b] The apparent ratios of octahedral to tetrahedral centers were estimated by serial dilutions of fixed concentrations of T- and R-state adducts. Previously published extinction coefficients were used to calculate the apparent distribution of octahedrally and tetrahedrally coordinated Co(II) complexes.

Methods. Insulin monomer concentrations were determined from absorbance measurements at 280 nm using a value of $\epsilon_{280} = 5700\ M^{-1}\ cm^{-1}$ (Porter, 1953). Cobalt-substituted insulin hexamers were prepared by the addition of Co^{2+} ions to buffered solutions of metal-free insulin in the ratio of 2 Co^{2+} ions/hexamer in 50 mM Tris-ClO_4 buffer, pH 8.0. Electronic spectra were recorded on a Hewlett-Packard 8450A spectrophotometer. CD spectra were recorded on a Jasco J-600 spectropolarimeter. Titration isotherms were performed by monitoring the absorbance changes at the λ_{max} of the d → d envelope for each complex as increasing concentrations of the phenol analogue were incorporated into the Co(II)–T_6 solution. Half-saturation points, $[S]_{0.5}$, and the Hill coefficients were calculated from plots of the logarithmic form of the Hill equation,[2] $\log[Y/(1-Y)] = n\log[A] - \log K$, where $Y = \Delta abs_{obs}/\Delta abs_{total}$. The apparent dissociation constants (K_D) for systems where $n_H = 1.0$ were calculated by fitting the ligand (A) titration curves to the hyperbolic equation $Y = [A]/(K_d + [A])$.

The apparent ratios of octahedral to tetrahedral centers in Table I were estimated by using previously published extinction coefficients to calculate the apparent amounts of the octa-

---

[2] Classically, Y has been defined as the fraction of ligands bound to the protein (Hill et al., 1925), but in this case, it is the fraction of tetrahedral metal sites. It is assumed that the structural transitions necessary to obtain a single tetrahedral metal site must be concerted in the trimer. This assumption seems reasonable when one considers that the Co(II) d → d transitions of four- and five-coordinate complexes are very sensitive to the geometry of the complex (Brader et al., 1991; Lever, 1986). Consequently, the species Co(II)–T_5R_1, Co(II)–T_4R_2, Co(II)–T_2R_4, and Co(II)–T_1R_5 almost certainly would give spectra significantly different from those of Co(II)–T_6, Co(II)–T_3R_3, and Co(II)–R_6. Because only a single tetrahedral species is detected in each of the equilibration titrations, we conclude that two Co(II) complexes dominate the absorption spectra, the octahedral T-state and the tetrahedral R-state. Therefore, we propose that [tetrahedral sites] $\propto$ [R_3 protomers] and that T_6, R_6, and/or T_3R_3 are the only significant species present in our titrations. Kaarsholm et al. (1989) reached similar conclusions about the significance of Zn(II)–T_6, Zn(II)–T_3R_3, and Zn(II)–R_6 from kinetic studies.

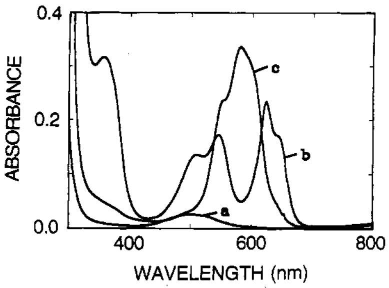


Figure 1: Electronic absorption spectra of 0.34 mM Co(II)–insulin hexamer measured in the absence of phenol and inorganic anions (a), in the presence of 60 mM phenol (b), and in the presence of 100 mM NaCl and 60 mM phenol (c).

hedral- and tetrahedral-coordinated Co(II) complexes in solution (Brader et al., 1991). The following apparent extinction coefficients, ϵ_{622} = 410 M^{-1} cm^{-1}, ϵ_{580} = 610 M^{-1} cm^{-1}, and ϵ_{572} = 750 M^{-1} cm^{-1}, were used to calculate the apparent concentrations of Co(II)–phenolate, Co(II)–Cl, and Co(II)–SCN R-state complexes.

RESULTS

Effects of Inorganic Anions on the T to R Allosteric Transition. Substitution of Co(II) for Zn(II) at the His-(B10) sites provides a useful optical probe for studying the T- to R-state conformational transition of the insulin hexamer (Roy et al., 1989; Brader et al., 1990, 1991; Brader & Dunn, 1990; Gross & Dunn, 1992). Because the coordination geometry of the Co(II) center undergoes conversion from octahedral to tetrahedral during the T- to R-state transition, the intensities and the energies of the Co(II) d → d transitions are substantially altered. The octahedral Co(II)–T_6 complex (Figure 1) is characterized by a broad, featureless d → d envelope of low intensity (λ_{max} = 495 nm, ϵ_{max} = 45 M^{-1} cm^{-1}), whereas the tetrahedral Co(II)–R_6 complexes are characterized by much more intense d → d bands with profiles that are very sensitive to the identity of the fourth ligand. The d → d absorptions of these Co(II)–R_6 complexes occur in the 540–630-nm region and possess intensities (ϵ_{max} > 300 M^{-1} cm^{-1}, normalized to metal ion concentration) that depend on ligand structures [see also Brader et al. (1990, 1991)].

Panels A and B of Figure 6, respectively, show the electronic absorption spectra and the circular dichroism (CD) spectra of the Co(II)–insulin hexamer in the presence of increasing SCN^- concentrations. From close inspection of these spectra, it is evident that these SCN^- solutions contain at least two Co(II) species, an octahedral species exhibiting a broad featureless envelope of d → d transitions with $\lambda_{max} \simeq$ 495 nm and a tetrahedral species with a much more intense envelope with λ_{max} = 572 nm. Upon addition of SCN^-, the octahedral Co(II)–T_6 complex is converted to a mixture of octahedral and tetrahedral species.[3] As estimated from the amplitude of the 572-nm band, the proportion of tetrahedral species formed appears to saturate at about 40%. The binding isotherm is hyperbolic in shape (Brader et al., 1991). Thus, unlike the phenolate adducts, the isotherm for the binding of SCN^- to the Co(II)–insulin hexamer is noncooperative.

The spectral changes observed for the equilibrium binding titrations described in Figure 1 indicate that the transition involves the conversion of the Co(II)–T_6 chromophore from an octahedral geometry to a new pseudotetrahedral geometry. This new pseudotetrahedral geometry corresponds to that of the Co(II)–R_6 complex stabilized both by the binding of the phenolic compound to the protein pockets and by the particular anion coordinated to the metal ion. Comparison of spectra obtained at intermediate degrees of saturation gave no indication of the presence of more than one tetrahedral species at equilibrium. Figure 1 also shows the spectrum of the Co(II)–R_6 hexamer measured in the presence of 100 mM phenol (spectrum b) and in the presence of both 100 mM Cl^- and 100 mM phenol (spectrum c). Brader et al. (1991) have assigned spectrum b of Figure 1 to the Co(II)–R_6 complex in which the phenolate ion is coordinated at the fourth ligand position. Spectrum c corresponds to that of the Cl^- adduct (Roy et al., 1989; Brader et al., 1990, 1991). The phenolate adduct gives a distinctive splitting of the d → d transitions into two envelopes with λ_{max} = 544 and 622 nm, whereas the Cl^- adduct exhibits a less structured envelope of bands with λ_{max} = 580 nm. The implications of these spectra for the respective Co(II) geometries have been previously discussed (Brader et al., 1991).

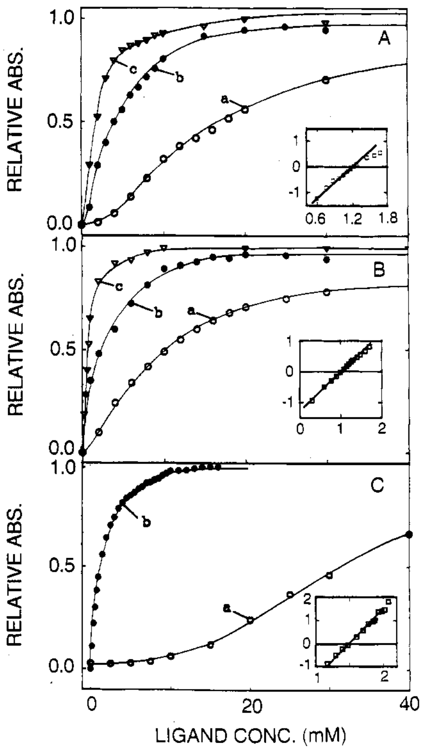


Figure 2: (A) Normalized binding isotherms for the titration of 0.34 mM Co(II)–insulin hexamer with *m*-cresol at pH 8.0 measured in the absence of inorganic anions (a), in the presence of 100 mM NaCl (b), and in the presence of 400 mM KSCN (c). The inset shows the Hill plot for a *m*-cresol titration in the absence of inorganic anions (n_H = 1.8). (b) Normalized binding isotherms for the titration of 0.34 mM Co(II)–insulin hexamer with resorcinol at pH 8.0 measured in the absence of inorganic anions (a), in the presence of 100 mM NaCl (b), and in the presence of 400 mM KSCN (c). The inset shows the Hill plot for a resorcinol titration in the absence of inorganic anions (n_H = 1.2). (C) Normalized binding isotherms for the titration of 0.34 mM Co(II)–insulin hexamer with 4-hydroxybenzamide at pH 8.0 measured in the absence of inorganic anions (a) and in the presence of 400 mM KSCN (b). The inset shows the Hill plot for 4-hydroxybenzamide titration measured in the absence of inorganic anions (n_H = 2.8).

Anions Act as Positive Allosteric Effectors. Figure 2A shows the isotherms for the binding of *m*-cresol to the Co(II)-substituted insulin hexamer at pH 8.0 in 50 mM Tris-ClO_4 buffer under the following conditions: (a) in the absence of inorganic anions, (b) in the presence of a saturating concentration of Cl^- (100 mM), and (c) in the presence of a

---

[3] Cyanate ion has been found to behave similarly (Brader et al., 1991; data not shown). Certain other monovalent anions (e.g., Cl^-, Br^-, I^-, and N_3^-) induce traces of the Co(II) R-state hexamer in the absence of phenolic ligands (W. E. Choi, P Brzovic', N. C. Kaarsholm, and M. F. Dunn, manuscript in preparation).

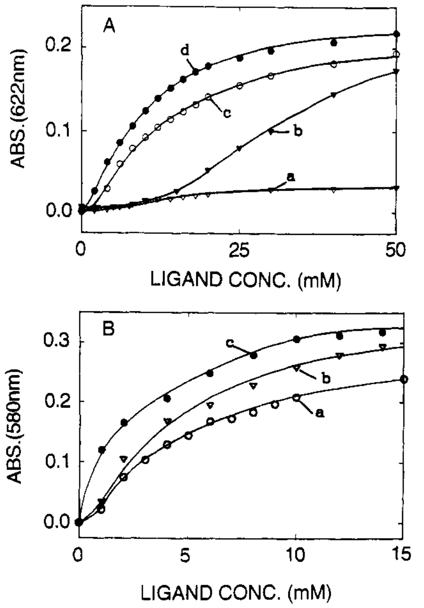


FIGURE 3: (A) Binding isotherms for the titration of 0.34 mM Co(II)–insulin hexamer with different phenolic compounds determined in the absence of inorganic anions: (a) *m*-cresol, (b) 4-hydroxybenzamide, (c) phenol, and (d) resorcinol. (B) Binding isotherms for the titration of 0.34 mM Co(II)–insulin hexamer with different phenolic compounds measured in the presence of 100 mM NaCl: (a) *m*-cresol, (b) phenol, and (c) resorcinol.

saturating concentration of SCN$^-$ (400 mM). The perchlorate ion from the buffer does not bind under these conditions. Therefore, in the absence of other organic anions, the fourth coordination position is occupied by the phenolate anion. Note that the equilibrium binding titration shown in Figure 2A, curve c, corresponds to the conversion of the SCN$^-$-induced mixture of octahedral and tetrahedral species (viz. Figure 6) to the Co(II)–R$_6$–SCN adduct induced by the binding of *m*-cresol. Therefore, the spectral contributions induced by SCN$^-$ prior to the addition of *m*-cresol have been subtracted from the changes resulting from the combined effects of SCN$^-$ and *m*-cresol. In the absence of added inorganic anions, the titration isotherm obtained with *m*-cresol is sigmoidal in shape (n_H = 1.8; $[S]_{0.5}$ = 16 mM)[2], indicating that the system is allosteric and possesses positive cooperativity (Monod et al., 1965; Koshland, et al., 1966). In the presence of 100 mM Cl$^-$, the titration isotherm (b) becomes less sigmoidal (n_H = 1.5; $[S]_{0.5}$ = 3 mM)[2] but displays increased binding affinity. In the presence of SCN$^-$, the titration isotherm is well-described by the equation for a rectangular hyperbola with an apparent K_D = 1.72 mM.

The overall sigmoidicity in the equilibrium binding titrations is most apparent in the absence of added inorganic anions; this is shown by the binding curves presented in Figure 3A from *m*-cresol (a), 4-hydroxybenzamide (b), phenol (c), and resorcinol (d). Since these analogues all give R-state spectra almost identical in shape (but not in amplitude, see below) to the phenolate ion complex (Figure 4), the spectral changes for these binding isotherms were measured at 622 nm (*m*-cresol, phenol, resorcinol) or at 618 nm (4-hydroxybenzamide). These analogues give sigmoidal isotherms similar to that of phenol in the absence of inorganic ions, with the 4-hydroxybenzamide binding curve showing the most pronounced sigmoidicity (n_H = 2.8; $[S]_{0.5}$ = 30 mM)[2] while the resorcinol binding curve shows the least pronounced sigmoidicity (n_H = 1.2; $[S]_{0.5}$ = 9 mM).[2] Given the underlying assumption that the d → d transitions of these phenolate adducts have similar molar extinction coefficients, the most striking difference between these analogues is the large variation in apparent yield of the Co(II)–R$_6$ state attained at saturating concentrations. Resorcinol is more effective in shifting the equilibrium in favor of the R-state than is phenol, *m*-cresol, or 4-hydroxybenzamide. Although the 4-hydroxybenzamide and *m*-cresol binding titrations give isotherms suggesting more cooperativity, these compounds appear to be much less effective in shifting the equilibrium toward the R-state than phenol or resorcinol. This effect may be a consequence of strong homotropic effects by 4-hydroxybenzamide and *m*-cresol coupled to the weaker heterotropic effects by the anionic form of the ligands. A similar effect (albeit somewhat reduced) is also observed in the presence of Cl$^-$ (Figure 3B) where, in comparison to phenol and resorcinol, the *m*-cresol-induced transition seems incomplete. A slightly weaker binding affinity of these phenolic compounds for the R-state may contribute to the incomplete conversion to the R-state hexamer. The 4-hydroxybenzamide titration was not included because 100 mM Cl$^-$ was insufficient to completely displace the phenolate anion from the fourth ligand coordination site of the metal.

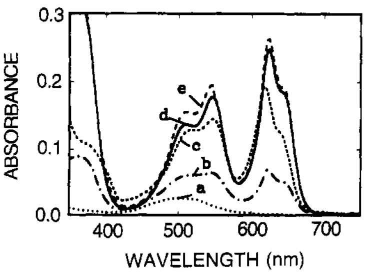


FIGURE 4: Electronic absorption spectra of 0.34 mM Co(II)–insulin hexamer in 50 mM Tris-ClO$_4$ buffer, pH 8.0 measured in the presence of 60 mM *m*-cresol (b), 90 mM 4-hydroxybenzamide (c), 120 mM phenol (d), and 65 mM resorcinol (e). Spectrum a is that of 0.34 mM Co(II)–insulin hexamer in buffer only.

The heterotropic effects of the Co(II)-substituted insulin hexamer (Brader et al., 1991) exhibit K-type allosteric system characteristics, where the binding of one ligand (an effector) modifies the apparent affinity of the protein for the second (heterotropic) ligand, and conversely (Monod et al., 1965). Figure 2B shows the effect of increasing resorcinol concentration on the binding isotherms of a 0.34 mM Co(II)–insulin hexamer solution both in the absence of inorganic anions and in the presence of Cl$^-$ or SCN$^-$. Unlike *m*-cresol, in the absence of inorganic anions, resorcinol (Figure 2B) is very effective in shifting the distribution in favor of the R-state. As shown by the decreased $[S]_{0.5}$ value, in the presence of Cl$^-$, the hexamer binds resorcinol more tightly than *m*-cresol. Figure 2C shows the effect of SCN$^-$ on the 4-hydroxybenzamide binding isotherms.

Determination of the Number of Phenol Binding Sites. Figure 5 shows the isotherm for the binding of 2,7-dihydroxynaphthalene (DHN) to 0.34 mM Co(II) insulin hexamer in the presence of 100 mM NaCl. The changes in the Co(II) d → d spectral bands show that DHN binds to the protein pockets of the insulin hexamer and, in the presence of 100 mM Cl$^-$, gives a Co(II)–R$_6$ chloride ion adduct. In the presence of NaCl, DHN binds very tightly to the protein pockets. This finding has allowed us to estimate the number of binding pockets from the end point of the titration (Figure 5). The intersection between the initial slope and the tangent (with slope = 0) to the titration end point yields a value of approximately 6 DHN binding sites/hexamer. In the absence of added anions, DHN gives an R-state complex with an envelope of d → d transitions similar to those previously identified as phenolate adducts (Brader et al., 1991; viz. Figure 1).

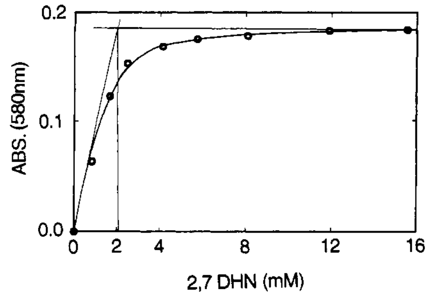


FIGURE 5: Binding isotherms for the titration of 0.34 mM Co(II)–insulin hexamer with 2,7-dihydroxynaphthalene determined in the presence of 100 mM NaCl. The intersection between the initial and final slopes provides an estimate of the number of binding sites for the hexamer.

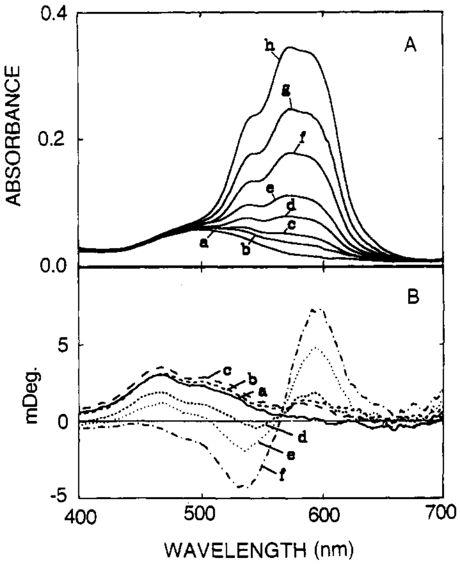


FIGURE 6: (A) Electronic absorption spectra of 0.68 mM Co(II)–insulin hexamer determined in the presence of increasing concentrations of KSCN [0 (a), 10 (b), 20 (c), 40 (d), 60 (e), 80 (f), 320 (g), and 640 mM (h)]. Note the significant contribution from the octahedral Co(II) center with envelope λ_{max} = 500 nm. (B) Circular dichroism spectra of 0.68 mM Co(II)–insulin hexamer with increasing KSCN concentrations: (a) 0, (b) 10, (c) 20, (d) 80, (e) 320, and (e) 640 mM.

DISCUSSION

T to R Allosteric Transition of the Insulin Hexamer. The two most widely applied models for allostery are the concerted model (MWC model) proposed by Monod et al. (1965) and the sequential model proposed by Koshland et al. (1966). The two-state concerted model is based on the premise that the allosteric transition occurs between two oligomeric forms of the same symmetry (T and R) which differ in affinity for the ligand. This model is defined by three adjustable parameters (the equilibrium constant L_0 for interconversion of the ligand-free T- and R-states and the dissociation constants for ligand binding to each state, K_T and K_R). Because the MWC model is constrained to two different quaternary conformations, each conformation must have a homogeneity of intersubunit interactions and binding sites. Therefore, each subunit in a given quaternary state must have the same conformation (Matthews & Bernhard, 1973; Seydoux et al., 1974). One limitation of this model is its inability to explain negative homotropic interactions (Conway & Koshland, 1968; Corwin & Fanning, 1968). The sequential model invokes ligand-dependent site–site interactions between subunits which lead to sequential protein structural changes as ligand is bound to the protein. In the general case, the sequential model contains m conformations (where m = the number of ligand binding sites) and a unique ligand dissociation constant for each conformation, thereby giving a more flexible, multiparameter model. This model can explain both positive and negative homotropic interactions, but examples of true negative homotropic allosteric systems are rare (Matthews & Bernhard, 1973). Both the concerted and the sequential models are extremes of a more general model described by Eigen (1967).

An alternative model which provides a possible explanation for the allosteric properties of the insulin hexamer and the existence of the T_6, T_3R_3, and R_6 states is the model proposed by Matthews and Bernhard (1973) and Seydoux et al. (1974), the SMB model.[4] This model postulates that the 2-fold symmetry often found in oligomers composed of subunits with identical covalent sequences (Matthews & Bernhard, 1973) gives a prevalence of even-numbered oligomers that are characterized by what Seydoux et al. (1974) define as "suboptimal intramolecular symmetry". Thus, the SMB model, with "pair-wise asymmetry in subunit conformation", assumes that (1) suboptimal symmetry can give rise to site heterogeneity and (2) there can be conformational isomerization among quaternary states of optimal and suboptimal symmetries. Depending on model parameters, the SMB model is capable of explaining both positive and negative cooperativity. In the extreme, this model predicts that half-site reactivity is a consequence of the assembly of the oligomer from asymmetric dimeric units.

Hemoglobin has been the classic subject of studies to investigate the mechanism of allosteric transitions (Baldwin, 1975; Baldwin et al., 1979; Perutz et al., 1987; Perutz, 1989). The refined, high-resolution X-ray structures of Zn(II)–T_6, Zn(II)–T_3R_3, and Zn(II)–R_6 make the insulin hexamer a compelling new example for the investigation of allostery. In each of the three crystallographically characterized types of insulin hexamer (T_6, T_3R_3, and R_6), the insulin subunits are arranged as three symmetry-related asymmetric dimers arranged about the 3-fold axis. Because each dimer is asymmetric, there are two different subunit conformations in each hexamer. The conformational differences in the T_6 and R_6 hexamers that give rise to asymmetry in the dimeric unit are small, whereas in the T_3R_3 hexamer, these differences are quite large. In the T state, residues 1–8 of the B-chain take up an extended conformation, while residues 9–19 takeup an α-helical conformation (Chart I). In the R_6 state, the B-chain α-helix extends from B1 through B19. Derewenda et al. (1989) and Smith and Dodson (1992a,b) have shown that phenol and phenol analogues stabilize the crystalline Zn(II)–R_6 hexamer by binding to six essentially identical hydrophobic pockets. The residues which form these pockets include portions of both A-chain helices and a portion of the B1–B8 helix from a subunit of the adjacent dimer. In the T-state, each of these pockets is partially filled by the side chain of Leu B6. Model-building studies using the coordinates for the crystal structure of the T_6 hexamer (Baker et al., 1988) indicate that the pockets are too sterically occluded to accommodate phenol.

---

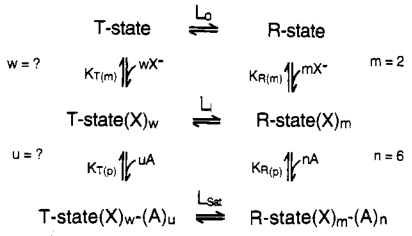
[4] The structures of several zinc insulin hexamers complexed to phenolic compounds, chloride ion, or thiocyanate ion have been found to be T_3R_3 species (Smith et al., 1984; Derewenda et al., 1991; Smith & Dodson, 1992a,b; G. D. Smith, private communication; G. G. Dodson, private communication). The prevalence of crystalline T_3R_3 species suggests that the stability of the T_3R_3 state is not a crystal lattice packing artifact but rather a manifestation of structural asymmetry arising from the assembly of the hexamer from asymmetric TR dimers.

The R_6 crystal structures show that there is a narrow tunnel along the 3-fold symmetry axis from the surface to each His(B10) metal site (Derewenda et al., 1989; Smith & Dodson, 1992a,b). The dimensions of this tunnel vary with the structure of the exchangeable ligand coordinated to the metal ion, indicating that the R_6 conformation must be fairly flexible. Brader et al. (1991) have shown that ligands which bind to the fourth ligand position on the tetrahedral metal center can provide important stabilizing interactions that favor the R-state. The equilibrium binding isotherms presented in Figure 2 establish that the Co(II)-substituted insulin hexamer exhibits behavior characteristic of a positive cooperative allosteric system responding to the binding of positive allosteric effectors (Monod et al., 1965; Koshland et al., 1966). Since there is little or no positive cooperativity between the two metal sites in the hexamer (Brader et al., 1991), the apparent positive cooperativity (viz. Figure 2) must arise from homotropic interactions among the six phenol-binding pockets of the insulin hexamer.

Partial Conversion Induced by SCN^- Anions. In this study, SCN^- is the only anion able to provide significant stabilization of a tetrahedrally coordinated complex *in the absence of cyclic organic molecules.*[3] The shape of the Co(II) d → d absorption envelope and the energies of the component transitions induced by SCN^- alone are essentially identical to those obtained in the presence of SCN^- and phenol, but the amplitude of this envelope is considerably lower. Consequently, SCN^- alone is capable of stabilizing a tetrahedrally-coordinated Co(II) complex in the absence of phenolic compounds. Close inspection of Figure 6A shows that, at saturating levels of SCN^-, the solution contains a mixture of octahedral and tetrahedral Co(II)-substituted hexameric insulin species.[2] This mixture of the two metal complexes is also evident in the circular dichroism spectra of the SCN^- titration (Figure 6B). It has been proposed that SCN^- stabilizes the T_3R_3 species in solution (Karrsholm et al., 1989; Thomas & Wollmer, 1989). We speculate that either SCN^- binds to both the T-state and the R-state hexamers, thereby stabilizing coexistent octahedral and tetrahedral systems, or SCN^- forms an asymmetric T_3R_3 complex that is a relatively stable species.[4]

The Allosteric Constant, L_{sat}, Depends on the Structure of the Phenolic Ligand. The equilibrium binding titrations performed with various phenol analogues (Figure 3A) give sigmoidal binding curves.[2] The DHN titration measured in the presence of 100 mM NaCl indicates a site stoichiometry of 6 DHN binding sites/hexamer, and the spectrum establishes that the tetrahedral coordination sphere of each metal ion is completed by a chloride ion.

Scheme I: Simplified Concerted Model[a]



[a] This model postulates interconverting T- and R- states of the insulin hexamer and ligand binding equilibria between neutral ligands which bind to the six hydrophobic pockets of the R-state (A) and anionic ligands (X^-) which coordinate to the Co^{2+} metal ions. X^- indicates a singly charged anionic ligand (heterotropic effector bound to the metal site), and A indicates a neutral organic ligand (homotropic effector bound to the phenolic protein pocket). The designations **w** and **m** correspond to stoichiometries of anionic ligand binding to the T- and R-state metal ions, respectively, while **u** and **n** are stoichiometries of neutral ligand binding to the T- and R-state hydrophobic pockets.

The majority of the crystallographic evidence (Baker et al., 1988; Derewenda et al., 1989; Smith et al., 1984; Smith & Dodson, 1992a,b), previous solution studies (Roy et al., 1989; Brader et al., 1991; Gross & Dunn, 1992), and the studies presented herein are consistent with a mechanism for the T_6 to R_6 interconversion that is best described by a model adapted either from the concerted model of Monod et al. (1965)[2] or the structural asymmetry model of Seydoux et al. (1974).[4] As is the case for the hemoglobin allosteric transition (Perutz, 1989), the fine details of crystallographic structure for the different R-state ligand complexes indicate that structurally different ligands give complexes with slightly different protein structures (presumably a reflection of the plasticity of the protein). The presence of three distinctly different hexamer structures (T_6, T_3R_3, and R_6) implies that any concerted model must include at least three protein conformational states. However, for the purposes of the following discussion, we adopt a simplified concerted model (Scheme I) to facilitate the analysis of our experimental observations. We believe presentation of more detailed models is premature. In Scheme I, ligands that bind to the hydrophobic protein pockets of the R_6 hexamer are designated as A, and singly charged anionic ligands that bind via coordination to the His(B10) metal ions are designated as X^-. The scheme postulates interconverting T- and R-states and ligand binding equilibra for A and X^-. Because the importance of T_3R_3 to this scheme has not been resolved,[4] we do not specify oligomer subunit conformations, nor do we define the stoichiometries of effector binding to the T-state. While the various X-ray structures provide detailed information about the binding loci and stoichiometries for the R-state hexamers, there is little information available about the loci and stoichiometries of the hypothesized ligand binding sites on the T-state. Each of the T-state octahedral His(B10) metal sites is a likely effector site for the binding of up to three monovalent anionic ligands. Dunn et al. (1980), Kaarsholm and Dunn (1987), and Kaarsholm et al. (1989) have shown that tridentate chelators can form transient ternary complexes with the Zn(II)– and Co(II)–T_6 hexamers in which the chelator is coordinated to the metal ion (presumably by displacement of the three water molecules coordinated to the octahedral metal ion). The T_6 hexamer has exposed hydrophobic surfaces that could form binding sites for neutral hydrophobic ligands (Blundell et al., 1972; Baker et al., 1988).

Although the phenolate adducts obtained with phenol and the phenol analogues give spectra with highly similar Co(II) d → d bands, the observed amplitudes are very different (viz. Figure 4). We think it is unlikely that these large differences in amplitude arise from large differences in the extinction coefficients. Although we also think it unlikely, it is possible that these differences arise from a situation where the complexes contain both tetrahedral and octahedral metal centers even though all the insulin subunits take up R-state conformations. At present, we are unable to eliminate this possibility. Herein, we assume that T_6 and R_6 are synonymous with octahedral and tetrahedral metal centers,[2] respectively. It then follows that differences in the amplitudes of the spectra must represent differences in the amount of tetrahedral adduct and/or R-state hexamer stabilized by the different phenolic compounds and inorganic anions. In the absence of effectors, the fraction of preexisting insulin-bound tetrahedral Co(II) is below the level of detection. Consequently, we estimate that the initial allosteric constant L_0 = [T-state]/[R-state] > 100 (Scheme I). In the presence of exogenous anions such as Cl^-, a new allosteric constant, L_1, is established[3] and the binding of some (but not all) of the phenolic compounds strongly shifts the equilibrium in favor of the R-state (Scheme I). The titration curves shown in Figure 3 are consistent with the conclusion that the different ring-substituted phenolic compounds give different values for the overall allosteric constant, L_{sat}, the equilibrium constant for interconversion of the T- and the R-states under conditions of site saturation (Table I). In this case, the proportion of R-state species stabilized at ligand saturation is dependent on the chemical structure of the ligand.

The monoclinic Zn(II)–R_6 crystal structure shows that, when bound to the protein pocket, the phenol ring makes van der Waals contact with the ring of His(B5), and the phenolic hydroxyl hydrogen-bonds both to the amide N–H of Cys(A11) and to the carbonyl oxygen of Cys(A6) (Derewenda et al., 1989). Different ring substituents undoubtedly modify some of these contacts (G. D. Smith, personal communication). The free energy change associated with inner-sphere coordination of Cl^- or SCN^- to give tetrahedral Co(II) adducts shifts the equilibrium in favor of the R-state (Scheme I) and the titration isotherms show decreased sigmoidicity *according to* the expected strength of binding of these positive effectors. As shown by the lower $[S]_{0.5}$ values, the apparent affinities of the phenolic ligands for the hexamer are increased by these heterotropic interactions. Among the inorganic anions investigated (Brader et al., 1990, 1991), SCN^- is the most effective in shifting the distribution of the two states in favor of the R-state in the presence of phenolic ligands.[3]

Both homotropic and heterotropic effects are superimposed in these titrations. All of the phenolic compounds examined in this study are capable of binding both to the hydrophobic pockets *and* to the fourth coordination site of the R-state Co(II) center, and the relative affinities of phenolic compounds for these two classes of sites can be quite different. Consequently, since tight binding to the metal site has a positive heterotropic effect on binding to the hydrophobic pockets (viz. Figure 2), the differences in relative affinities necessarily impose different degrees of sigmoidicity[2] in these isotherms. Using the concerted model (Monod et al., 1965) for illustrative purposes, then for homotropic interactions, the model predicts that as the value of c (where $c = K_R/K_T$) decreases, the Hill coefficient, n_H, increases. Thus, if a structural change in the phenolic ligand causes tighter binding to the R-state hydrophobic pocket, then the titration curve will appear more sigmoidal, whereas the more tightly a positive heterotropic effector binds to the R-state metal site, the more hyperbolic the curve becomes, causing n_H to tend toward a value of 1.0. According to this analysis, the more sigmoidal character of the 4-hydroxybenzamide binding isotherm in Figure 3A (*vis â vis* phenol) and the apparent lower yield of the Co(II)–R_6 4-hydroxybenzamide phenolate adduct (Figures 3A and 4 and Table I) must reflect a smaller value of c for binding to the hydrophobic pockets that is not offset by tighter binding to the R-state metal sites. [Similar arguments would apply either to a suitably tailored sequential model (Koshland et al., 1966) or to the model, based on pairwise asymmetry, of Seydoux et al. (1974).] Therefore, to explain the low yield of the R-state species formed in the presence of a saturating concentration of *m*-cresol, we postulate that the *m*-cresolate ion establishes a new L_1, favoring the stabilization of the T-state (Scheme I). Comparison of the spectra of the resorcinol and *m*-cresol complexes (Figure 3A) indicates that for resorcinol the final equilibrium $L_{sat} \simeq 0.11$, whereas for *m*-cresol $L_{sat} \simeq 6$ (Table I). The redistribution of T- and R-states induced by these phenolic compounds depends both on the different ring-substituted chemical groups of the phenolic compounds and on the nature of the exogenous anion present in solution. Inspection of Table I indicates that the relative affinities of the different phenolic compounds for Co(II)–T_6 and Co(II)–R_6 must be quite different.

Heterotropic Interactions Stabilize the R-State Hexamer. Inorganic anions and phenolate anions bind to the fourth coordination site of the Co(II)–R_6 species, whereas only the neutral phenolic derivatives bind to the six protein pockets of the hexamer. The heterotropic interactions between these two loci can significantly stabilize the R-state [viz. Figure 2 and Brader et al. (1991)]. Both Cl^- and SCN^- ions appear to increase the apparent affinity [$K_{R(p)}$] of phenolic ligands for the hexamer by changing the allosteric constant L_1 (Figure 2 and Scheme I) and therefore behave as K-system heterotropic allosteric effectors (Monod et al., 1965). These titrations establish that the extent to which ligand binding stabilizes the R-state depends both on the nature of the substituents on the phenol ring and on the nature of the anion coordinated to the metal ion.

In summary, it is well established that the Co(II)-substituted insulin hexamer undergoes a phenol-induced T_6 to R_6 transition in solution (Roy et al., 1989; Thomas & Wollmer, 1989; Kaarsholm et al., 1989; Brader et al., 1990, 1991; Kruger et al., 1990; Brader & Dunn, 1991; Gross & Dunn, 1991). The significance of the T_3R_3 species in solution has not been settled (Kaarsholm et al., 1989; Kruger et al., 1990; Brader et al., 1991). The binding of phenol and other phenolic compounds favors the R-state of the hexamer by driving the conformational equilibrium toward the R-state through stabilizing van der Waals contacts and hydrogen bonds which alter the relative ground-state energies of both the T- and the R-state conformations of the insulin hexamer. These studies establish that binding of phenolic ligands to the protein pockets is a positive cooperative homotropic effect, while inorganic anions such as Cl^-, SCN^-, and the anionic forms of the phenolic ligands stabilize the R-state through heterotropic interactions between the metal site and the phenolic pockets. In the absence of phenolic ligands, SCN^- shifts the T to R equilibrium such that the ratio of octahedral to tetrahedral metal centers is approximately 0.6. We conclude that SCN^- alone does not bring about the complete transformation of T to R because either SCN^- binds to both states with similar affinities, thus yielding an equilibrium which slightly favors the T-state at saturation, or there is an inherent structural asymmetry in the hexamer that renders T_3R_3 a relatively stable state.[4]

ACKNOWLEDGMENT

We thank Webe Kadima and Peter Brzovic for helpful discussions, David Smith for providing the coordinates for the rhombohedral R_6 structure, and David Smith and Guy Dodson for communicating structural information about various T_3R_3 complexes prior to publication. We also thank Sheng-Tung Huang for performing some of the titrations.

REFERENCES

Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N., & Vijayan, N. M. (1988) *Philos. Trans. R. Soc. London, B 319*, 369–456.

Baldwin, J. M. (1975) *Prog. Biophys. Mol. Biol. 29*, 225–320.

Baldwin, J. M., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Bentley, G. A., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., & Mercola, D. A. (1976) *Nature 261*, 166–168.

Blundell, T. (1979) *Trends Biochem. Sci. 4*, 51–54.

Blundell, T., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279–402.

Brader, M. L., & Dunn, M. F. (1990) *J. Am. Chem. Soc. 112*, 4585–4587.

Brader, M. L., & Dunn, M. F. (1991) *Trends Biochem. Sci. 16*, 341–345.

Brader, M. L., Kaarsholm, N. C., & Dunn, M. F. (1990) *J. Biol. Chem. 265*, 15666-15670.

Brader, M. L., Kaarsholm, N. C., Lee, W.-K., & Dunn, M. F. (1991) *Biochemistry 30*, 6636–6645.

Brader, M. L., Borchardt, D., & Dunn, M. F. (1992a) *J. Am. Chem. Soc. 114*, 4480–4486.

Brader, M. L., Borchardt, D., & Dunn, M. F. (1992b) *Biochemistry 31*, 4691–4696.

Coffman, F. D., & Dunn, M. F. (1988) *Biochemistry 27*, 6179–6187.

Conway, A., & Koshland, D. E., Jr. (1968) *Biochemistry 7*, 4011–4023.

Corwin, L. M., & Fanning, G. P. (1968) *J. Biol. Chem. 243*, 3517–3525.

Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Reynolds, C. D., Smith, G. D., Sparks, C., & Swensen, D. (1989) *Nature 338*, 594–596.

Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Bing, X., & Markussen, J. (1991) *J. Mol. Biol. 220*, 425–433.

Dodson, E. J., Dodson, G. G., Hodgkin, D. C., & Reynolds, C. D. (1979) *Can. J. Biochem. 57*, 469–479.

Dunn, M. F., Pattison, S. E., Storm, M. C., & Quiel, E. (1980) *Biochemistry 19*, 718–725.

Eigen, M. (1967) *Nobel Symp. 5*, 333–369.

Gross, L., & Dunn, M. F. (1992) *Biochemistry 31*, 1295–1301.

Hill, C. P., Dauter, Z., Dodson, E. J., Dodson, G. G., & Dunn, M. F. (1991) *Biochemistry 30*, 917–924.

Hill, R. (1925) *Proc. R. Soc. B100*, 419.

Kaarsholm, N. C., & Dunn, M. F. (1987) *Biochemistry 26*, 883–890.

Kaarsholm, N. C., Ko, H.-C., & Dunn, M. F. (1989) *Biochemistry 28*, 4427–4435.

Koshland, D. E., Nemethy, G., & Filmer, D. (1966) *Biochemistry 5*, 365–385.

Krüger, P., Gilge, G., Cabuk, Y., & Wollmer, A. (1990) *Biol. Chem. Hoppe-Seyler 371*, 669–673.

Lever, A. B. P. (1986) *Inorganic Electronic Spectroscopy*, Elsevier Publishing Co., New York.

Matthews, B. W., & Bernhard, S. A. (1973) *Annu. Rev. Biophys. Bioeng. 2*, 257–315.

Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.

Peking Insulin Structure Research Group (1974) *Scient. Sin. 17*, 779–792.

Perutz, M. F. (1989) *Q. Rev. Biophys. 22*, 139–237.

Perutz, M. F., Fermi, G., Luisi, B., Shaanan, B., & Liddington, R. C. (1987) *Acc. Chem. Res. 20*, 309–321.

Porter, R. R. (1953) *Biochem. J. 53*, 320–328.

Roy, M., Brader, M. L., Lee, R. W.-K., Kaarsholm, N. C., Hansen, J., & Dunn, M. F. (1989) *J. Biol. Chem. 264*, 19081–19085.

Sakabe, N., Sakabe, K. & Sasaki, K. (1981) in *Structural Studies on Molecules of Biological Interest* (Dodson, G., Glusker, J. P., & Sayre, D., Eds.) pp 509–526, Clarendon Press, Oxford, England.

Schichtkrull, J. (1958) *Insulin Crystals*, Munsgaard, Copenhagen, Denmark.

Seydoux, F., Malhotra, O. P., & Bernhard, S. A. (1974) *CRC Crit. Rev. Biochem. 2*, 227–257.

Smith, G. D., & Dodson, G. G. (1992a) *Biopolymers 32*, 441–445.

Smith, G. D., & Dodson, G. G. (1992b) *Proteins: Struct., Funct., Genet. 14*, 401–408.

Smith, G. D., Swenson, D. C., Dodson, E. J., Dodson, G. G., & Reynolds, C. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7093–7097.

Storm, M. C., & Dunn, M. F. (1985) *Biochemistry 24*, 1749–1756.

Sudmeier, J. L., Bell, S. J., Storm, M. C., & Dunn, M. F. (1981) *Science 212*, 560–562.

Thomas, B., & Wollmer, A. (1989) *Biol. Chem. Hoppe-Seyler 370*, 1235–1244.