Conformations of Carp Muscle Calcium Binding Parvalbumin†

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ABSTRACT: Of the two Ca(II) in carp muscle calcium binding parvalbumin B, one may be removed by dialysis against EGTA without significant alteration of the circular dichroism spectra at 224 nm, a result suggesting little or no change in the helical content of 47%. Binding of the two Ca(II) is not cooperative. Addition of a 20-fold or greater excess of EGTA at pH 8.5 results in removal of both Ca(II), reduces the helical content to about 39%, alters only slightly the tertiary structure as indicated by proton magnetic resonance, and frees the single cysteine residue so that it reacts more rapidly with a sulfhydryl reagent. Lanthanide ions bind more strongly than and substitute for Ca(II) with little or no change in ultraviolet circular dichroism. Luminescence of the terbium(III) protein at 545 nm results upon irradiation in the phenylalanine region at 259 nm. Energy transfer from the aromatic side chain of Phe-57 to Tb(III) at the Ca(II) site in the EF loop accounts for the luminescence, which is also circularly polarized. Tb(III) fluorescence also appears upon irradiation of rabbit muscle troponin C in the tyrosine absorption region. In troponin C, Tyr-108 is homologous with Phe-57 in the parvalbumin, and a similar proximity of the aromatic ring to the Ca(II) binding site is suggested.

The white muscles of fishes and amphibians contain significant amounts of a class of highly soluble proteins, called parvalbumins, which possess molecular weights of only 9000–13,000, isoelectric points from pH 4 to 5, and about 9% phenylalanine residues (Pechere *et al.*, 1971b; Hamoir and Konosu, 1965; Konosu *et al.*, 1965). Carp parvalbumin consists of three electrophoretically distinguishable components; the middle one, designated B, is the subject of this research. For carp parvalbumin B the complete sequence of 108 amino acids (Coffee and Bradshaw, 1973) and the three-dimensional conformation to 1.85-Å resolution have been determined (Kretsinger and Nockolds, 1973). The protein contains no tyrosine or tryptophan, only one each of histidine, arginine, and cysteine, and ten phenylalanine residues that are not stacked. Its molecular weight of 11,489 includes two calcium ions. Carp parvalbumin is folded and ellipsoidal in shape and contains six helical regions of similar length, designated A–F. Loops occur between the helical regions. The AB loop contains the single cysteine residue, the CD loop one Ca(II), the DE loop the single arginine residue, and the EF loop the other Ca(II). We designate the two Ca(II) sites as Ca(CD) and Ca(EF). Only the latter appears accessible to solvent. An approximate twofold symmetry axis relates the C,D and E,F helical regions and the two Ca(II) distorted octahedra. Each Ca(II) is bound only by oxygen donors, four of which are negatively charged carboxylate oxygens of aspartate and glutamate side chains. The binding strength of the two Ca(II) have been measured in hake and frog parvalbumins and stability constants of 2.5 to 10×10^6 reported by Benzonana *et al.* (1972), who also suggested that the two Ca(II) may be bound cooperatively.

In this paper we report results of the behavior of carp muscle calcium binding parvalbumin B in aqueous solutions under a variety of conditions. Our investigation centers on the relation between calcium binding and conformation.

Materials and Methods

Carp muscle calcium binding parvalbumin B was prepared and purified as described by Kretsinger and Nockolds (1973). Protein concentration was determined by utilizing a molar absorptivity of ϵ 2000 at the 259-nm maximum. This molar absorptivity agrees with that found for hake parvalbumin, which also contains ten phenylalanine residues (Pechere *et al.*, 1971a), and that reported for *N*-acetylphenylalaninamide (Barel and Glazer, 1969). Troponin C was kindly supplied by Dr. Cyril Kay (Murray and Kay, 1972) to whom we are grateful.

All chemicals were high grade commercial products. EGTA[1] experiments were usually conducted at pH 8.5 in a 0.1 M buffer of trishydroxymethylaminomethane (Tris). In a typical dialysis experiment 10^{-4} M protein in the presence of 10^{-3} M Tris buffer and 0.1 M KCl was dialyzed against a solution containing the last two reagents and 10^{-3} M EGTA. Then the protein solution containing EGTA was dialyzed against a solution containing buffer and salt. Ca(II) analyses were performed in the trace analysis laboratory of this department on a Perkin-Elmer Model 303 atomic absorption spectrophotometer over a range of 1–10 ppm of calcium. Standards which contained the same buffers and salts as the unknowns were utilized. Proton magnetic resonance spectra were obtained on a JEOL PS-100P FT spectrometer. Positive chemical shifts are quoted in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate. The reaction with 2 mM Nbs_2 was followed at 412 nm with a protein concentration of about 0.1 mM. Nd(III) and Tb(III) were added as chlorides. The concentration of Nd(III) in the MCD experiments was 5 mM. Circular dichroism and Faraday effect (MCD) spectra were taken on a Jasco J10 B instrument. The Tb(III) fluorescence titration was conducted at pH 6.5 in piperazine buffer and 0.1 M KCl. No enhancement of Tb(III) fluorescence was observed in Tris buffer at pH 8.5 or bis-tris buffer at pH 6.5.

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[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.

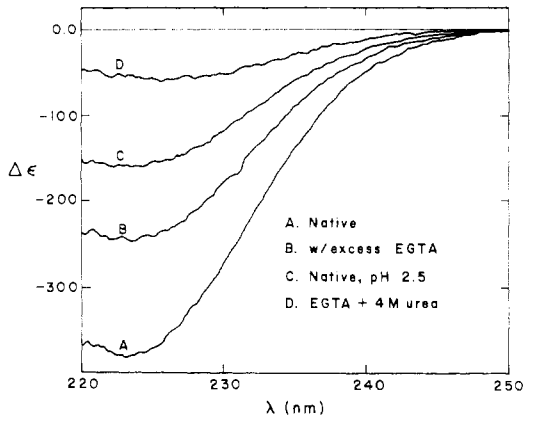

FIGURE 1: Circular dichroism spectra of carp parvalbumin under a variety of conditions. Curves A, B, and D are at pH 8.5. The ordinate scale is the differential molar absorptivity per mole of protein.

Fluorescence and circularly polarized luminescence (CPL) experiments of parvalbumin were performed on an apparatus designed and built in this department by F. S. Richardson and C. K. Luk. The protein concentration was about 0.3 mM. During the 2 hr required to record the CPL spectrum shown in Figure 2 the protein suffered radiation damage, as evinced by appearance of shoulders at 280 and 300 nm in the absorption spectrum. Phenylalanine is photosensitive (Hasselmann and Laustriat, 1973). The fluorescence of troponin C was measured on a Perkin-Elmer MPF-3 spectrometer through the courtesy of Professor C. Huang. Except for some dialyses which took place at 4°, all experiments were conducted at room temperature, about 23°.

Results

With ten phenylalanine residues as the only strongly absorbing aromatic amino acid, carp parvalbumin B exhibits vibrational structure in the 250–270-nm absorption region which was useful in monitoring the state of the protein. In carp parvalbumin C a tyrosyl replaces a phenylalanyl residue in position 2 and as a result only it gives appreciable absorption at wavelengths longer than 280 nm. These absorption spectra are similar to those shown for hake parvalbumins (Pechere *et al.*, 1971a,b). The circular dichroism spectrum of carp parvalbumin B is shown in Figure 1, curve A, and appears qualitatively similar to that of hake parvalbumin (Parello and Pechere, 1971) and to that expected for a protein with appreciable α-helical content. The CD minimum at 224 nm shown in Figure 1 was used in monitoring the conformation.

Ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA) forms a strong Ca(II) complex and the effect of the addition of the ligand on the solution properties of the parvalbumin was followed by circular dichroism (CD) and absorption spectra. The extent of spectral changes depends upon the pH. All the experiments described in this paragraph were performed within 0.5 hr. At pH 6 even a 100-fold excess of EGTA produces no change in the CD magnitude at 224 nm. At pH 8.5 a reduction in magnitude of the minimum at 224 nm in the CD spectrum of native protein upon addition of excess EGTA levels off when the molar ratio is about 20. The resulting CD spectrum is shown in Figure 1, curve B, and is obtained for EGTA to parvalbumin molar ratios from 20 to 100 or more. The phenylalanine bands from 250 to 270 nm in the absorption spectrum undergo a shift of about 0.5 nm to shorter wavelength upon addition of EGTA. At pH 9.9 a lesser amount of excess EGTA is sufficient to reduce the CD magnitude to the same level as curve B in Figure 1. All the above spectral changes are completely reversible upon addition of Ca(II) to the solution.

A series of dialysis experiments where the conformation of the parvalbumin was monitored by CD and the Ca(II) content by atomic absorption spectroscopy relates CD magnitudes at 224 nm with Ca(II) content of the protein. By atomic absorption the sample of native protein used contained 2.4 equiv of Ca(II)/mol of protein; the additional 0.4 equiv is presumably nonspecifically bound Ca(II). By dialyzing 10^{-4} M protein against 10^{-3} M EGTA at pH 8.2 at room temperature for periods shorter than 0.5 hr, the Ca(II) content of the parvalbumin solution was reduced to 1.2 equiv of Ca(II) while the CD spectra remained close to that of native protein (curve A in Figure 1). Ca(II) contents less than 1 equiv correlated with reduced CD magnitudes until, after dialysis for about 1 day, curve B in Figure 1 is obtained with a Ca(II) content near 0 equiv in the parvalbumin solution.

The 100-MHz proton magnetic resonance spectrum of a solution with 50 mg/ml of protein consists of broad generally featureless bands that one associates with native proteins. A small peak was observed at −0.4 ppm and an about ten times larger one downfield at +0.2 ppm similar to those depicted by Parello *et al.* (1974). Peaks in this very high field range are usually attributed to ring current shifts (Sternlicht and Wilson, 1967; McDonald and Phillips, 1967), in this case to juxtaposition of methyl groups near the aromatic face of phenylalanine residues. Addition of a 30-fold excess of EGTA at pH 8.5 resulted in loss of the small peak at −0.4 ppm, but a peak at +0.2 ppm remained. Only small changes occurred elsewhere in the spectrum.

Treatment of native parvalbumin at pH 8.5 with 4 M urea reduces somewhat the CD magnitude at 224 nm to $\Delta\epsilon$ = 290 from 370. If, however, one first adds excess EGTA as shown by curve B in Figure 1 and then makes the solution 4 M in urea, the result is an additional reduction in CD magnitude to yield curve D in Figure 1. The combination of EGTA and 4 M urea also shifts the phenylalanine absorption bands 1 nm to shorter wavelengths. Parvalbumin that is dialyzed against EGTA and 4 M urea at pH 8.9 has a Ca(II) content of nearly 0 equiv and may be returned to the native state as measured by the CD at 224 nm by the addition of Ca(II).

The presence of base up to pH 11 has little effect on the CD at 224 nm or the phenylalanine absorption bands. Acid, however, reduces the CD magnitude at 224 nm until at pH 3 it is less than that produced by excess EGTA at pH 8.5 (curve C in Figure 1). At pH 3 the phenylalanine absorption bands are shifted 1 nm to the blue. Down to pH 2.5 the effects of added acid are reversible by addition of base. At about pH 2 the carp parvalbumins become unstable and precipitate from solution. Increasing the temperature to 60° at neutral pH produces little or no change in the CD and absorption spectrum of native protein.

Accessibility of the single sulfhydryl group was measured by reaction with excess Nbs_2 (Means and Feeney, 1971) at pH 8.5 in 0.1 M Tris buffer. Development of additional absorption at 412 nm was immediate in the presence of 30-fold excess EGTA in the presence or absence of 4 M urea. The reaction with native protein was slower: the half-life for development of full absorption was about 1.5 min. Thus the sulfhydryl group is less accessible in the native protein than

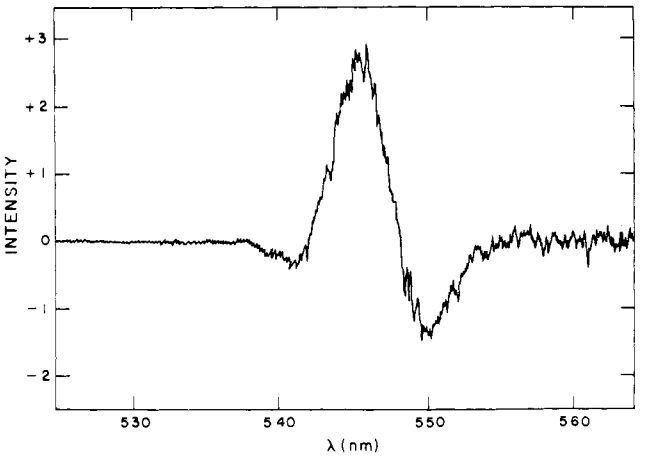

FIGURE 2: Circularly polarized luminescence spectrum of 0.6 mM Tb(III) with irradiation of 0.3 mM carp parvalbumin at 259 nm. Intensity units are arbitrary.

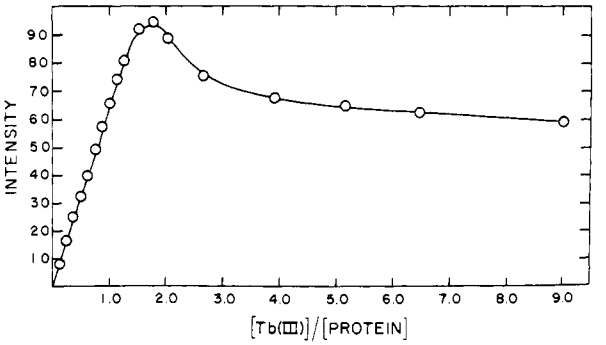

FIGURE 3: Fluorescence titration of parvalbumin at 545 nm upon addition of Tb(III) at pH 6.5. Intensity units are arbitrary.

in the presence of excess EGTA.

Trivalent lanthanide ions have been increasingly substituted for Ca(II) in proteins as the two kinds of metal ions possess similar diameters and a preference for binding to ligands with oxygen donor atoms (Prados *et al.*, 1974). Compared to their sharp f–f absorption and circular dichroism spectra, which are both weak, trivalent lanthanide ions exhibit relatively strong and complicated magnetic circular dichroism (MCD) spectra. Distinctive visible MCD spectra of Nd(III) bound to water and two amino acid ligands have been reported (Sipe and Martin, 1974). The MCD spectra of solutions containing parvalbumin and a constant concentration of Nd(III) in 12:1 and 2.5:1 mole ratios were measured in this research. For controls the MCD spectra of solutions containing the same weight ratio of the latter solution were run on bovine albumin and lysozyme. Absorption spectra of the f–f transitions were not observed in the protein solution because of the low concentration of Nd(III). The results indicate that both control proteins and the 12:1 solution of parvalbumin exhibit visible MCD spectra similar in sign pattern and relative intensities to that reported for aqueous Nd(III). In these solutions most of the Nd(III) exists as the aqueous ion. On the other hand, the solution containing a 2.5:1 molar ratio of parvalbumin to Nd(III) displayed a uniquely different MCD spectrum of weak intensity. This result is consistent with a large fraction of the Nd(III) present replacing Ca(II) in the protein.

The fluorescence properties of the trivalent lanthanide Tb(III) were exploited to ascertain properties of the two Ca(II) sites in parvalbumin. Addition of equimolar amounts of Tb(III) to solutions of native parvalbumin and excitation of Tb(III) absorption at 365 nm did not produce visible fluorescence in a darkened room. Upon changing the exciting wavelength to 259 nm, where only phenylalanine absorption bands appear, a strong green fluorescence with a major peak at 545 nm and smaller peaks at 490, 590, and 625 nm, characteristic of Tb(III), was observed. The CD and ultraviolet absorption spectra employed as monitors of conformation in the above studies were unchanged upon addition of Tb(III). Due to its asymmetric environment in a Ca(II) site, the green fluorescence from the Tb(III) complex of parvalbumin is also circularly polarized, as shown in Figure 2. Instead of the single peak at 545 nm found in the fluorescence spectrum, the circularly polarized luminescence exhibits three distinct peaks and potentially provides more information. No circularly polarized luminescence is observed in similar experiments when Tb(III) is added to bovine albumin at pH 6.5. That the Tb(III) fluorescence with parvalbumins is reduced by addition of excess Ca(II) to the solution suggests the existence of a facile equilibrium between the metal ions for a site in the protein.

The effects of added increments of Tb(III) on the fluorescence intensity at 545 nm upon irradiation of native parvalbumin at 259 nm are shown in Figure 3. The curve reaches a maximum at a Tb(III) to protein molar ratio of 1.8 with a decrease in intensity at higher ratios perhaps due to complexing of Tb(III) on the surface of the negatively charged protein. Dialysis of 2×10^{-4} M parvalbumin against 10^{-3} M Tb(III) at pH 6.3 in 0.01 M piperazine buffer gave a Ca(II) content near zero and a CD curve close to that of native protein. These two kinds of experiments suggest that both Ca(II) of parvalbumin are replaceable by Tb(III).

To provide additional evidence concerning Tb(III) binding, crystals were prepared for an X-ray diffraction study. $TbCl_3$ was added to a solution containing 50 mg/ml of native parvalbumin until the molar ratio was 2:1. The resulting solution had a pH of 6.8. Protein crystals were grown by slowly diffusing in ammonium sulfate until the solution was 73% saturated. From the X-ray diffraction results a vector map was constructed, utilizing as coefficients the differences between the structure factors of the Tb(III) containing crystal and a native crystal (Moews and Kretsinger, 1974). Difference synthesis with both native protein and refined phases was also performed. The results indicate a high occupancy of the Ca(EF) site by Tb(III), two secondary surface sites with some Tb(III), and virtually no Tb(III) in the Ca(CD) site. Because of the high concentration of $(NH_4)_2SO_4$, a final pH near 5, the long period of standing, and the possible introduction of additional Ca(II), the relation between this crystal structure analysis and the solution results is uncertain. However, the crystal structure result indicates the isomorphism of Ca(II) replacement by Tb(III) in the EF site and does not support the suggestion of cooperative binding.

Fluorescence experiments were also performed on the addition of about 1 equiv of Tb(III) to rabbit muscle troponin C in 0.01 M piperazine buffer at pH 6.3. Upon irradiation in the tyrosyl absorption region at 280 nm (troponin C contains no tryptophan) some quenching of protein fluorescence at 310 nm occurs and Tb(III) fluorescence at 545 nm appears. The excitation spectrum for Tb(III) fluorescence at 545 nm shows a maximum at 280 nm, implicating a tyrosyl residue in the energy transfer process. Addition of a

second equivalent of Tb(III) produces only a small increment in fluorescence intensity. Thus the first equivalent of Tb(III) added to troponin C gives rise to a pronounced energy transfer from a tyrosyl residue to Tb(III).

Discussion

Based on the circular dichroism standards derived from proteins at 224 nm ($[\theta]$ = 37,000 for helical and +11,000 for random structures, Saxena and Wetlaufer, 1971), the α-helical content of the native parvalbumin is 47% (curve A, Figure 1). This value is in excellent agreement with the X-ray structure determination which indicates that close to half of the residues are found in helical regions. Thus an excellent correspondence appears to exist between the native conformation in aqueous solutions and that in the crystal.

Addition of excess EGTA to pH 8.5 solutions of parvalbumin reduces the CD absorption as shown by curve B in Figure 1, and the calculated α-helical content becomes 39%. This reduction corresponds to disruption of one of the six helical regions found in the native protein. The presence of excess EGTA induces only small changes in the proton magnetic resonance spectrum of the protein: most of the high field resonances indicative of proximity of methyl groups to the faces of phenylalanine residues are little affected. Thus EGTA seems to disrupt the equivalent of one helical region with maintenance of most of the tertiary structure.

The limited conformational changes induced by EGTA are presumably due to its Ca(II) binding capability. The dialysis experiments where both Ca(II) content and CD were monitored indicate that native parvalbumin with 2 equiv of Ca(II) and a protein with about 1 equiv of Ca(II) give rise to similar CD curves that appear as curve A in Figure 1. Evidently one Ca(II) may be removed from parvalbumin with little or no change in helical content. This result suggests that binding of the two Ca(II) is not cooperative. We suggest that the easily removed Ca(II) is the one in the EF loop.

Curves B and D (and presumably C) in Figure 1 correlate with removal of nearly all Ca(II) from the protein. Thus removal of both Ca(II) with EGTA may yield a CD curve that corresponds to loss of only one of six helical regions. Hence Ca(II) does not seem to be required for maintenance of most of the parvalbumin conformation. These results suggest a functional role for Ca(II) in parvalbumin beyond that of maintenance of protein integrity.

Addition of EGTA with Ca(II) removal results in more rapid reaction of the single cysteine residue of carp parvalbumin with a sulfhydryl reagent. The guanidinuim group of arginine-75 donates a hydrogen bond to the carbonyl oxygen of cysteine-18 in the AB loop (Kretsinger and Nockolds, 1973). Modification of the single arginine residue in a pike parvalbumin by reaction with 1,2-cyclohexanedione resulted in halving of the Ca(II) content from two to less than one per molecule of protein (Gosselin-Rey *et al.*, 1973) and a reduction in helicity from 48 to 40% corresponding again to the equivalent loss of one of six helical regions (recalculated from CD results at 222 nm as described above). These changes involve the single arginine residue that occurs in all parvalbumins examined and the single cysteine residue that occurs in all but one component of pike parvalbumin. Interestingly, an interplay between Ca(II) binding and sulfhydryl group reactivity occurs in a number of proteins with a variety of functions. These include α-amylase (Steer and Levitzki, 1973), S-100 protein (Calissano *et al.*, 1969), and a mammalian transglutaminase (Folk and Cole, 1966; Folk *et al.*, 1967). As with parvalbumin, removal of Ca(II) from α-amylase frees sulfhydryls to react with sulfhydryl reagents. On the other hand, removing Ca(II) from S-100 masks sulfhydryl reactivity. Transglutaminase requires Ca(II) in order to catalyze an acyl-transfer reaction where an active sulfhydryl group is essential for catalytic activity. This kind of interplay in proteins which are turned on or off by Ca(II) suggests that the agent responsible for transmitting the effect of Ca(II) to other proteins or substrates may be sulfhydryl groups.

The results reported with lanthanide ions indicate that they substitute for and bind more strongly than Ca(II) in carp muscle calcium binding parvalbumin. According to the three-dimensional structure of the protein (Kretsinger and Nockolds, 1973; Hendrikson and Karle, 1973) each Ca^{2+} binding site contains four carboxylate oxygen donors and should be able to accommodate the additional positive charge offered by trivalent lanthanide ions. The weakness of the Nd(III) transitions in the unique magnetic circular dichroism spectrum of the 2.5:1 parvalbumin solution suggests that some of the lanthanide ions occupied a site of high symmetry such as an octahedral one.

Terbium may be substituted for calcium to yield a CD similar to that of native parvalbumin. In Tb(III) substituted parvalbumin irradiation at 259 nm, where only protein absorbs, results in strong luminescence at 545 nm, where only Tb(III) emits. Since the oscillator strengths of Tb(III) transitions are so weak, the strong luminescence requires good proximity of the two groups involved in energy transfer. The only aromatic group near a Ca(II) site in the native parvalbumin is Phe-57, of which the carbonyl group serves as a donor to Ca (CD), but the side chain is within 5 Å of Ca (EF). Thus the energy transfer occurs between the aromatic side chain of Phe-57 and the Tb(III) substituted for Ca(II) in the loop.

Tb(III) fluorescence also appears upon substitution of the lanthanide for Ca(II) in troponin C. However, in this case the excitation spectrum identifies one of two tyrosyl residues in troponin C as involved in the energy transfer. Rabbit muscle troponin C is homologous with carp muscle calcium binding parvalbumin B, and in the alignment of the two amino acid sequences Phe-57 of the parvalbumin is matched with Tyr-108 in troponin C (Collins *et al.*, 1973). Thus sequence similarities between the fish and mammalian protein carry over to the conformations of the two proteins where it is suggested that the aromatic ring of Tyr-108 is close to a Ca(II) binding site.

Acknowledgments

This research would not have been possible without the cooperation and interest of several people. Professor R. H. Kretsinger supplied samples of the parvalbumin and provided expertise on its preparation. Conversations with him and Dr. P. Moews on the three-dimensional structure as determined by X-ray diffraction were invaluable. The circularly polarized luminescence results and features of their interpretation are the product of efforts by Professor F. S. Richardson and Dr. C. K. Luk. Dr. Cyril M. Kay supplied the sample of troponin C.

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Mechanism of Cooperative Oxygen Binding to Hemoglobin: Equilibrium Aspects†

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ABSTRACT: The sequential theory of D. E. Koshland, G. Nemethy, and D. Filmer [(1966), *Biochemistry 8,* 2580] is extended to explain the influence of 2,3-diphosphoglycerate and inositol hexaphosphate on the oxygen-binding properties of hemoglobin. The modification of the theory is made on the basis that organic phosphates can bind to hemoglobin molecules in intermediate stages of oxygenation and that the ligand affinities of the α and β chains differ from each other when the hemoglobin molecule forms a complex with the organic phosphate. This model is applied to equilibrium oxygenation studies of hemoglobin and is seen to accurately represent the published data with a minimum of parameters.

The molecular mechanism for the cooperative oxygenation of hemoglobin (Hb) has been a subject of intense research during the past 70 years and has also been used as a model for understanding the actions of regulatory enzymes. There are enormous amounts of experimental data in the literature on both equilibrium and kinetic studies of this protein (Huisman and Schroeder, 1971; Antonini and Brunori, 1971). Human adult hemoglobin is a protein molecule consisting of four subunits, namely two α chains with 141 amino acid residues each and two β chains with 146 amino acid residues each (Huisman and Schroeder, 1971). In 1910, Hill (1910) fitted the experimental data on the oxygenation of hemoglobin by using the empirical relations

$$\mathrm{Hb} + n\mathrm{O_2} \overset{k}{\rightleftharpoons} \mathrm{Hb(O_2)_n}$$

$$Y_{O_2} = kp^n/(1 + kp^n) \quad (1)$$

where Y_{O_2} is the fraction of oxygenation, k is the association constant, p is the partial pressure of oxygen, and n is the Hill coefficient which is an empirical measure of the cooperativity of the oxygenation of hemoglobin. For a protein molecule consisting of four subunits, the maximum value of n is 4. The Hill coefficient in eq 1 would be unity if the four subunits were identical and noninteracting and if there were no quaternary structural change in the protein molecule. The Hill coefficient for the oxygenation of hemoglobin has been found to be approximately 3 (Antonini and

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