Conformational Analysis by Nuclear Magnetic Resonance: Insulin†

Kenneth L. Williamson*,‡ and R. J. P. Williams*

ABSTRACT: High-resolution 270-MHz proton nuclear magnetic resonance (NMR) spectra of the native two-zinc insulin hexamer at pH 9 have been obtained, and assignments of key resonances have been made. Spectra of zinc-free insulin titrated with Zn^{2+} are unchanged after the addition of 1 equiv of zinc per insulin hexamer, indicating that the conformation of the hexamer is fixed at this point and that the second zinc ion does not significantly change the conformation. Titration of the two-zinc insulin hexamer with anions high on the Hofmeister series such as SCN^- causes marked changes in the NMR spectra which are interpreted as the result of major conformational changes to a new hexameric form of insulin having a twofold axis perpendicular to the threefold axis. Analysis of difference spectra indicates that this new hexamer (which should be capable of binding six zinc ions) binds 2 equiv of SCN^- at two sites which are assumed to be identical and independent ($K_1 = 10^3$, $K_2 = 2.5 \times 10^2$ M^{-1}).

The correlation of structure and function of insulin depends on a detailed chemical and physical characterization of the native hormone. The pioneering work by Sanger and his collaborators (Sanger et al., 1955; Ryle et al., 1955) elucidated the primary structure of insulin, and the X-ray crystallographic work of Hodgkin and co-workers (Adams et al., 1969; Blundell et al., 1972) has given us the secondary, tertiary, and quaternary structures of the native two-zinc crystalline hormone. Recently, Bentley et al. (1976) reported the X-ray structure of a second form of insulin, a slow-acting clinical preparation, which crystallizes with four zinc ions when the sodium and chloride ion concentration in the solution is 6% or more.

In this four-zinc form, as in two-zinc insulin, there are three equivalent dimers associated about the threefold axis to form a hexamer. In the two-zinc form there are two zinc ions on the threefold axis, attached through B10 histidine residues to the almost identical monomer molecules, I and II, of each insulin dimer. In four-zinc insulin one zinc ion is on the threefold axis attached to molecules nearly identical with molecule II of two-zinc insulin. The remaining three zinc ions occupy sites symmetrically disposed to the threefold axis and are linked to three molecules of I which are much changed in conformation at that end of the hexamer. The change is effected by a major rearrangement of the first eight residues of the B chain. This is represented diagrammatically in parts a and b of Figure 1 with views down the threefold axis shown in parts c and d of Figure 1.

Recently, a variant of the four-zinc structure has been found in refinement of the initial X-ray work (G. Dodson and G. Bentley, unpublished experiments). In this new form the three molecules of I coordinate just one zinc ion in the hexamer instead of three. This occurs by rotation of the B10 His side chain about the C_α–C_β bond from the position reported for four-zinc insulin (Bentley et al., 1976) so that it points toward the threefold symmetry axis. In this latter position the His coordinates one zinc ion (which is located on the threefold axis). However, except for the B10 His, the remainder of the conformation of the molecule is unchanged from that of four-zinc insulin. Both possibilities exist simultaneously in the crystal, and the relative amounts of the two forms depend critically on the crystallization medium. This is further evidence that it is the anion and not the zinc which causes the change in going from the two-zinc insulin structure to the four-zinc insulin structure.

The object of the present work was to study the conformations and transformations of these two forms of insulin *in solution* under conditions as near to physiological as possible by using high-resolution proton nuclear magnetic resonance (NMR) spectroscopy.

All previous proton NMR studies, because of the low solubility of insulin at neutral pH, have been carried out in solvents and under conditions where one would not expect the native hormone to exist. Kowalsky (1962) reported 56-MHz CW spectra at pH 2 and 10 by using 10 and 20% aqueous solutions. Bak, Pedersen, and co-workers recorded spectra of insulin dissolved in trifluoroacetic acid at 56 (Bak et al., 1967) and 220 (Bak et al., 1968) MHz. A ^{19}F NMR study of (trifluoroacetyl)insulins was carried out by Paselk & Levy (1974) at various pH values, and Led et al. (1975) have recently measured the carbon-13 spin–lattice relaxation times of 90% ^{13}C-enriched carbamyl groups bound to three sites on insulin.

The aggregation properties and conformations of insulin have been shown to be dependent on pH, salt concentration, metal ions, protein concentration, and temperature. On the basis of the equilibrium dialysis and sedimentation experiments of Goldman & Carpenter (1974), two-zinc insulin under our experimental conditions should be hexameric, as it is in the crystal.

Experimental Section

Insulin. Samples of zinc-free insulin [prepared according to the procedure of Schlichtkrull (1965)] and pure recrystallized two-zinc bovine insulin (containing 0.54% zinc) were obtained from J. Schlichtkrull. In a typical sample preparation 52 mg of crystalline two-zinc bovine insulin was dissolved/suspended in 1.0 mL of a 10% solution of CD_3COOD in 99.9% D_2O with warming on a water bath. To the white suspension was added a minimum quantity of 10% NaOD in D_2O in order to bring the pH to 8.9–9.1, at which point a perfectly clear, nonviscous solution 9 mM in insulin resulted. pH will refer throughtout to the actual reading on the predominantly D_2O solutions.

Titrations. For the zinc ion titrations appropriate quantities of 6 mM zinc sulfate in 6 mM zinc-free insulin at pH 9.5 were added by micropipet to the NMR sample tube containing a

† From the Department of Chemistry, Mount Holyoke College, South Hadley, Massachusetts 01075 (K.L.W.), and the Inorganic Chemistry Laboratory, Oxford OX1 3QR, United Kingdom (R.J.P.W.) *Received December 12, 1977; revised manuscript received June 28, 1979.* This work was supported by the National Institutes of Health (Grant GM 10224-13).

‡ John Simon Guggenheim Fellow.

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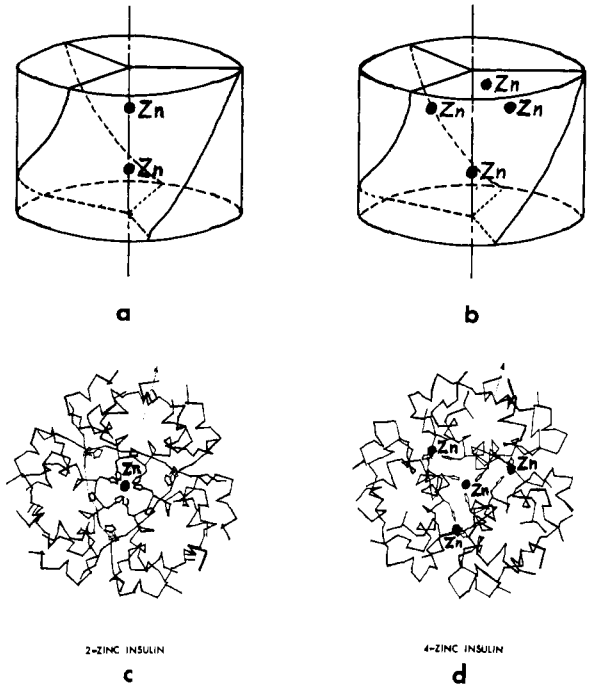

FIGURE 1: Diagrammatic representations of (a) two- and (b) four-zinc insulin. Views down the threefold axis of (c) two-zinc and (d) four-zinc insulin. Only α-carbon atoms, histidine side chains, and zinc ions are shown. The anion binding sites are on the threefold axis.

6 mM solution of zinc-free bovine insulin at pH 9.5. Similarly, for the thiocyanate titrations appropriate quantities of 2.0 M KSCN in 9 mM two-zinc bovine insulin at pH 9.1 were added to 9 mM two-zinc bovine insulin at the same pH. Spectra were usually run within minutes of mixing. The appearance of these spectra was shown to be independent of the time elapsed between mixing and collecting the free induction decays.

NMR Methods. ^{1}H NMR spectra were recorded on a FT 270-MHz Bruker spectrometer with an Oxford Instrument Co. superconducting magnet coupled to a Nicolet 1085 computer with a 293 pulse controller and a 294 disk system. The D_2O present in the samples served as a field-frequency lock. Irradiation of the proton resonance of the HDO present was carried out by the gated decoupling technique of Hoult & Richards (1975). Measurements were made at 27 °C, and chemical shifts are reported in parts per million downfield from 2,2-dimethyl-2-silapentane-5-sulfonate. Difference spectra were obtained according to the method of Campbell et al. (1975). Blank spectra (no added thiocyanate) did not shift on subtraction. It has previously been shown, using multiple internal blanks, that thiocyanate does not affect the lock; registration of spectra was indicated by lack of correspondence of difference spectra with either the original or the altered spectrum. Line widths are not altered upon the addition of thiocyanate.

Results

270-MHz ^{1}H NMR Spectrum of Two-Zinc Bovine Insulin. The 270-MHz ^{1}H NMR spectrum of two-zinc bovine insulin at pH 8.9 and a concentration of 9 mM is seen in Figure 2a. This spectrum is essentially identical with the one collected at pH 8.0 and a concentration of 4 mM, but which took over 4 times as long to obtain with a similar signal-to-noise ratio. On the basis of previous ^{1}H NMR studies on proteins by Roberts & Jardetzky (1970), Dwek (1973), Campbell et al. (1974, 1975), Dobson et al. (1975), Bradbury et al. (1973), and McDonald & Phillips (1967), we can assign many of the peaks in these spectra. These assignments are given in Table I. In the present work we shall be primarily concerned with two groups of peaks: those in the region from about 0.5 to 1.2 ppm which arise from the seventy-two protons of the twenty-four methyl groups of the one isoleucine, six leucines, and five valines in each insulin monomer and those in the region 6.7–7.7 ppm which arise from the thirty-five aromatic protons of the two histidines, three phenylalanines, and four tyrosines. Since all spectra are run in D_2O, the low-field (8–10 ppm) NH resonances are not seen. The ratio of the total aromatic peak area to the shaded peak area at 7.55 ppm is 35:1.04, indicating that the lowest field peak arises from one of the C(2)H imidazole protons of one of the two histidines present in the insulin monomer. The X-ray structure of two-zinc insulin hexamer shows a symmetrical structure in

Table I: 270-MHz ^{1}H Chemical Shifts of Major Peaks and Their Assignments for Two-Zinc Bovine Insulin

major peaks in insulin (ppm)[a]	reported ppm[b]	no. and type of amino acid protons	protons in insulin
0.59–1.11	0.92, 0.99	1 Ile CH_3 (2)	6
	0.95	6 Leu CH_3 (2)	36
	0.97, 1.02	5 Val CH_3 (2)	30
1.40	1.24, 1.45	1 Ile CH_2	2
	1.31	1 Thr CH_3	3
	1.46	3 Ala CH_3	9
	1.46	1 Lys γ-CH	1
1.67–1.75	1.67	1 Arg δ-CH_2	2
	1.70	6 Leu β-CH_2, γ-CH	18
	1.70	1 Lys δ-CH	2
1.88	1.87	1 Arg β-CH_2	2
	1.87	1 Lys β-CH_2	2
2.04	2.01	1 Pro γ-CH_2	2
	2.01	1 Ile β-CH	1
	2.06	4 Glu β-CH_2	8
	2.07	1 Pro β-CH	1
	2.10	3 Gln β-CH_2	6
2.32	2.25	5 Val β-CH	5
	2.32	4 Glu γ-CH_2	8
	2.32	1 Pro β-CH	1
	2.43	3 Gln γ-CH_2	6
2.91–2.97	2.85, 2.91	3 Asn β-CH_2	6
	2.85	4 Tyr β-CH_2	8
	3.02	1 Lys ϵ-CH_2	2
	3.05	6 Cys β-CH_2	12
3–5	3.10	4 Tyr β-CH_2	8
	3.11, 3.27	3 Phe β-CH_2	6
	3.18	2 His β-CH_2	4
	3.22	1 Arg δ-CH_2	2
	3.30	1 Pro δ-CH_2	2
	3.51	4 Gly α-CH_2	8
	3.51	1 Thr α-CH	1
	3.59	5 Val α-CH	5
	3.64	1 Ile α-CH	1
	3.69	6 Leu α-CH	6
	3.73	1 Lys α-CH	1
	3.74	1 Arg α-CH	1
	3.76	3 Ala α-CH	3
	3.82	3 Ser α-CH	3
	3.93	3 Ser β-CH_2	6
	3.95	6 Cys α-CH	6
	3.97	3 Asn α-CH	3
	3.98	3 Phe α-CH	3
	3.98	2 His α-CH	2
	4.11	1 Pro α-CH	1
	4.22	1 Thr β-CH	1
6.83	6.88	4 Tyr 2-ortho	8
7.15	7.08	2 His C(4)H	2
	7.20	4 Tyr 2-meta	8
	7.35	3 Phe 2H	6
	7.4	3 Phe 3H	9
7.56	7.84	2 His C(2)H	2

[a] See Figure 2. Shifts were measured in D_2O at 27 °C, pH 9.1. [b] McDonald & Phillips (1967). Shifts were reported in neutral D_2O at 40 °C.

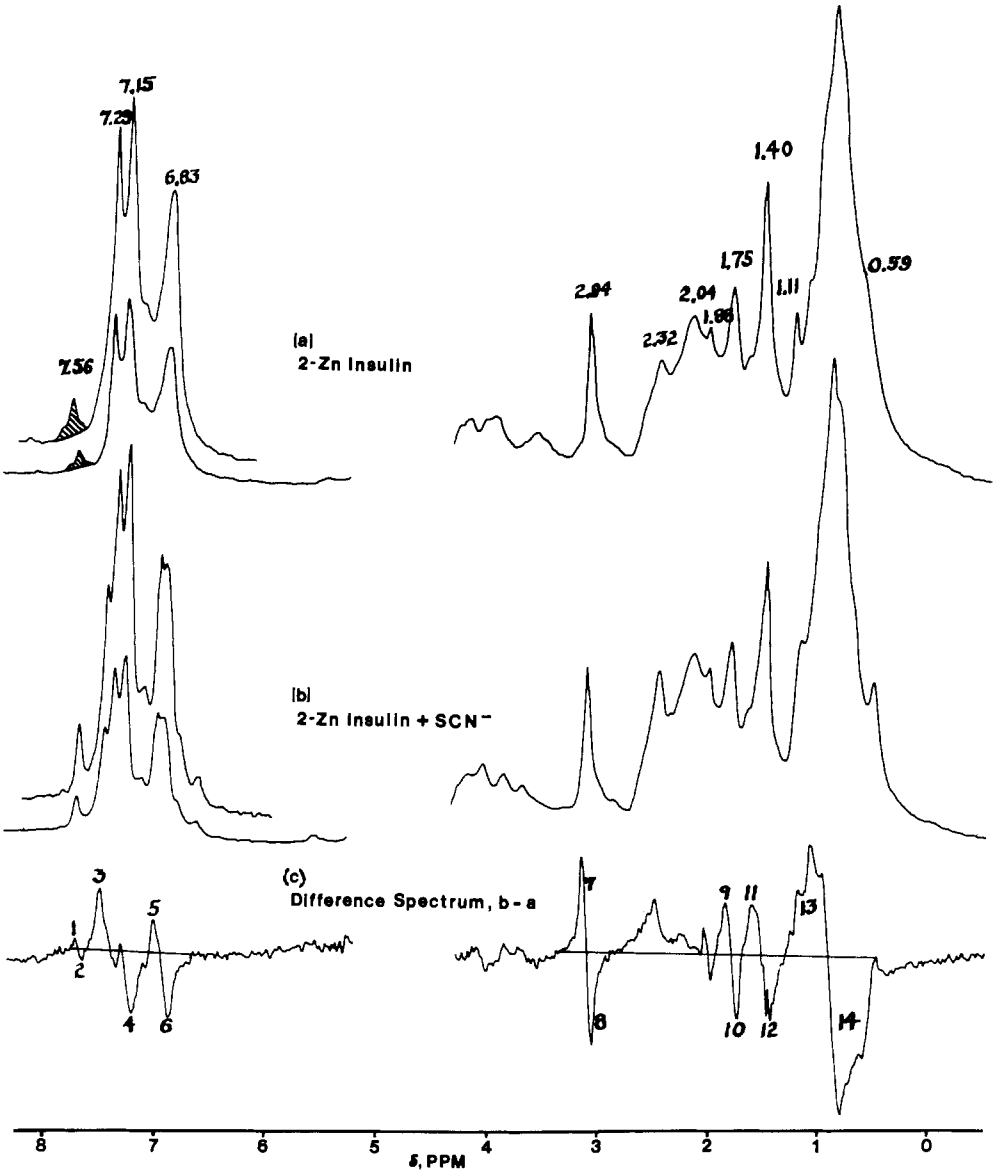

FIGURE 2: 270-MHz ^1H NMR spectrum of (a) two-zinc bovine insulin at pH 8.9 and a concentration of 9 mM and of (b) two-zinc bovine insulin at pH 8.9 and a concentration of 9 mM containing 2.0 mM SCN^-. (c) Difference spectrum (b − a).

which each insulin monomer has an almost identical conformation (within tenths of angstroms), so this peak must arise from six equivalent C(2)H protons on either the B5 or B10 histidine since at pH 9.1 and a concentration of 9 mM we expect, as noted above, that our sample will be hexameric in solution.

In hen egg white lysozyme the 3,5-ortho protons of three tyrosines have chemical shifts of 6.71, 6.83, and 6.98 ppm (Campbell et al., 1975). In the two-zinc insulin spectrum (Figure 2a) we can, by analogy, assign the 6.83-ppm peak to the same ortho protons of the four tyrosines, A14, A19, B16, and B26. Similarly, the 2,6-meta protons were found at 7.05, 7.09, and 7.24 ppm in lysozyme, and therefore we tentatively assign the 7.15-ppm peak in two-zinc insulin to these eight meta protons from the four tyrosine residues. The remaining peak at 7.29 ppm must be part of the broad multiplet arising from the fifteen protons on the three phenylalanines, B1, B24, and B25. In lysozyme rapid rotation or oscillation about the axis of the three tyrosines produces effective equivalence of the H_2 and H_4 protons and between the H_3 and H_5 protons. The evidence for this rapid rotation or oscillation, which would have a minimum rate of 10^4 s^{-1}, is not so clear in insulin. Two insulin tyrosines, A14 and A19, are on the outside of the hexamer and presumably could oscillate rapidly enough to become equivalent. The other two tyrosines are more or less buried, but spectroscopic and chemical evidence regarding their accessibility is somewhat contradictory (Blundell et al., 1972).

The small peak at ~6.9 ppm may arise from the C(4)H protons of one or two of the histidine residues.

Turning to the far upfield region, we find a broad and fairly featureless peak extending from the shoulder at 0.59 ppm to the small peak at 1.11 ppm, with the main peak centered at 0.75 ppm. In a random coil arrangement we would expect to find the twenty-four methyl groups of the isoleucine, the six leucines, and the five valines between 0.92 and 1.02 ppm (Table I). The greater range found in the two-zinc insulin hexamer reflects the deshielding (and shielding as well) of some of these methyls by aromatic rings. At present we cannot make specific assignments in this region, but it is noteworthy that no peaks are shifted far upfield.

Titration of Zinc-Free Insulin with Zn^{2+}. In Figure 3 are seen the spectra of 6 mM zinc-free insulin to which aliquots of 6 mM zinc sulfate (in 6 mM zinc-free insulin) have been added. The spectrum of zinc-free insulin at pH 9.5 is quite

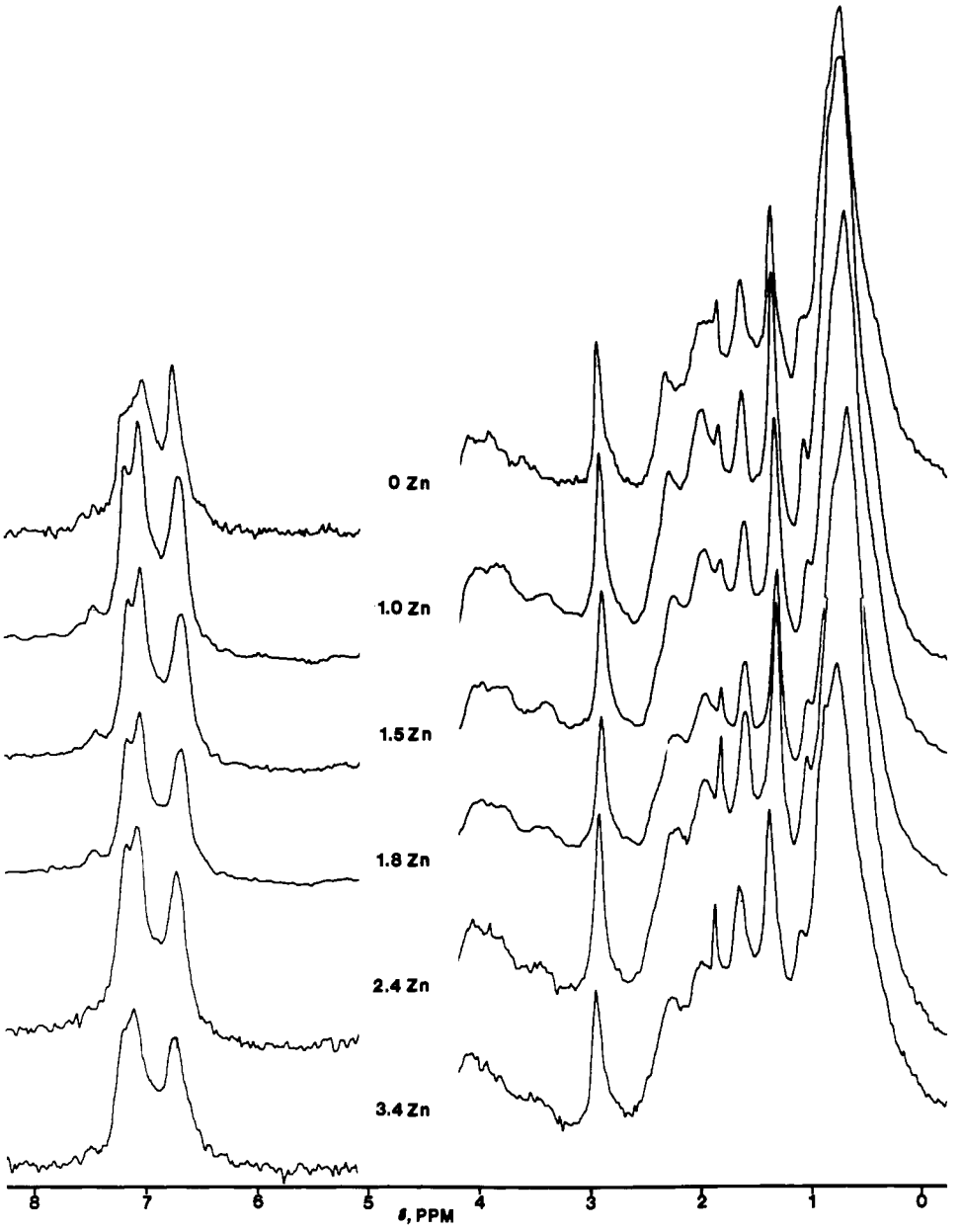

FIGURE 3: 270-MHz ^1H NMR spectra of zinc-free insulin (6 mM, pH 9.5) titrated with Zn^{2+}.

different from two-zinc insulin, particularly in the aromatic region. From equilibrium sedimentation experiments on zinc-free insulin at pH 8, Goldman & Carpenter (1974) concluded that dimer formation is much stronger than tetramer or hexamer formation. At the concentrations of insulin used to obtain the NMR spectra in Figure 3 (6 mM), we can expect to have mostly dimer with some tetramer and hexamer present. Addition of the first aliquot of Zn^{2+} causes a dramatic change in the aromatic region of the NMR spectrum (Figure 3). This amount of zinc corresponds to 0.167 g-atom of zinc per insulin monomer or 1 g-atom of zinc per insulin hexamer. Further additions of zinc cause very little change in the appearance of the spectra. Close inspection reveals an increase in line width at the higher Zn^{2+} concentrations which increases markedly with further additions of Zn^{2+} (up to 6 mM Zn^{2+}). This increase in line width is due to viscosity broadening. Ultracentrifuge experiments (Cunningham et al. 1955) on the addition of Zn^{2+} to zinc-free insulin show that the sedimentation constant rises from 2.7 S to ~3.5 S on the first addition of zinc. Further addition of zinc causes no further increase in $s_{20,w}$ until ~1.8–2.4 zinc atoms/hexamer are present. Above that point further addition of Zn^{2+} leads to a linear increase in $s_{20,w}$. Fredericq (1956) found a 72 000-dalton unit, and then, when the zinc concentration rises above six atoms over hexamer, a polydisperse distribution in the range of 200 000–300 000 daltons is found to predominate at pH 8. This aggregation of zinc insulin hexamers is due to general weak binding of Zn^{2+} to some 10–12 sites, probably neutral α-amino groups, with association constants of (7–35) $\times$ 10^3 M^{-1} (Goldman & Carpenter, 1974; Summerell et al., 1965).

Goldman & Carpenter (1974), on the basis of equilibrium dialysis experiments, measured apparent association constants of 1.86×10^5 and 7.3×10^3 M^{-1} for the two sites. This is in agreement with the work of Brill & Venable (1967), who first postulated the stepwise binding of zinc to insulin. It is suggested that the first zinc coordinates with the three insulin dimers in much the same way as to three independent small molecule ligands. With this first zinc in place the coordinating atoms of the second site have a relatively high probability of being in the proper position to bind the second zinc, and hence the association constant for the binding of the second ion will be greater than for the first.

The present work confirms this hypothesis. After only 1 equiv of zinc per hexamer is added to zinc-free insulin, the NMR spectra are essentially unchanged. This indicates that the conformation of the zinc hexamer, as reflected in the NMR spectra, is fixed by the binding to one zinc atom and that the binding of a second zinc atom does not change the conformation of the hexamer, again as reflected in the NMR spectra.

Treatment of Two-Zinc Insulin with SCN⁻. In the course of his long and careful studies on the crystallization of insulin, Schlichtkrull (1958) discovered that when insulin was crystallized from a medium containing more than 6% sodium chloride in the presence of 4 equiv of zinc the crystals isolated contained four zinc atoms per hexamer. The transition from two-zinc crystals to 4-zinc crystals is a sharp one, and no more than four zinc ions are incorporated even at sodium chloride concentrations of 15% in the presence of excess zinc. Schlichtkrull found further that four-zinc crystals could not be obtained in the absence of sodium chloride, even when the zinc ion concentration is very high. In fact, when 6 equiv of zinc is present in the crystallization medium, the crystallization will be very slow or will not take place at all.

It has recently been found (G. A. Bentley and A. Lewitovâ, unpublished experiments) that four-zinc insulin can be obtained with other anions in the crystallizing medium. With thiocyanate ion the crystals obtained are isomorphous with crystals obtained with chloride ion (those used for the X-ray analysis). Therefore, since insulin was much more soluble in the presence of added thiocyanate than chloride in these preparations, the effects of the former anion were examined more closely in the subsequent NMR studies.

Remarkable changes occur in the NMR spectrum of two-zinc insulin upon the addition of chloride ion. These same changes can be induced by lower concentrations of iodide ion and by even lower concentrations of thiocyanate ion. This order of anion activity is the same as the "lyotropic" series noted by Hofmeister (1888) in an examination of the effectiveness of anions upon the swelling of many proteins. The uptake of anions by biological systems has recently been critically reviewed by Frausto da Silva & Williams (1976). A quantitative study of the Hofmeister series has been done by Fridovich (1963), who examined anion binding to acetoacetic decarboxylase. He found that the main contribution to the binding of thiocyanate, which is at the top of the list, is the large enthalpy change in the reaction. Thiocyanate is not highly hydrated and can shed its water of hydration easily on electrostatic binding to a large and poorly hydrated cationic center in the protein.

Four-zinc insulin has been crystallized from iodide ion containing solutions (G. A. Bentley, S. M. Cutfield, and A. Lewitovâ, unpublished experiments). The iodide ion is found by X-ray crystallography to enter the hexamer on the threefold axis; presumably thiocyanate does the same.

In Figure 2b is the 270-MHz ^{1}H NMR spectrum of two-zinc insulin (9 mM, pH 9.1) containing 9 mM SCN^-. Comparison with the spectrum of two-zinc insulin seen under exactly the same conditions, but lacking the SCN^- (Figure 2a), reveals profound changes. These are easily seen in the difference spectrum in which the spectrum of two-zinc insulin is subtracted from the spectrum of two-zinc insulin to which has been added thiocyanate. Many peaks are subtracted out entirely; changes revealed in the difference spectrum must be attributed, then, to a change in the conformation of insulin caused by the thiocyanate and consequent changes in the chemical shifts of certain amino acid residues. The relative peak areas for the major peaks in the difference spectrum are given in Table II. These areas are given in numbers of protons per peak and are standardized against the whole aromatic peak area which represents 35 protons. As noted above, thiocyanate does not cause changes in the line widths or changes in the chemical shift of the D_2O peak, so the spectra are in register.

Table II: Peak Areas from Difference Spectrum

peak no.	area (no. of protons)	peak no.	area (no. of protons)
1	0.45	8	4.0
2	0.32	9	2.2
3	3.8	10	2.3
4	4.0	11	2.8
5	1.1	12	3.9
6	4.1	13	12
7	3.6	14	22

Although the X-ray crystal structure of rhombohedral crystals of two-zinc insulin indicates that it does not have a perfect twofold symmetry axis perpendicular to the threefold axis, it is clear that the conformations of molecules I and II which make up the dimer in hexameric two-zinc insulin are *very* similar. The two zinc atoms are 17 Å apart.

Upon the addition of SCN^-, a conformational change in the molecule occurs and four-zinc insulin can be crystallized from the medium. From the X-ray crystal structure it is seen that the number of contacts between the dimers of the hexamer drops from 108 to fewer than 40, while at the same time the number of coordinated zinc ions increases. Because of the symmetry of the hexamer about the twofold axis, it would seem that there might be the possibility that SCN^- could cause further disruption of the two-zinc conformation to give a completely symmetrical form of insulin capable of binding six zinc ions. While such a form would lose stability through loss of dimer–dimer contacts, it would gain stability through binding to a total of four new zinc ions and (see below) two thiocyanate ions.

Addition of chloride and iodide ions to two-zinc insulin also causes a gradual change in the NMR spectra, so that eventually they have the same appearance as that of Figure 2b, where thiocyanate has been added. On a mole per mole basis SCN^- is the most effective anion in effecting this change, but in each case the spectra ultimately produced are the same. As noted above, anions like Cl^-, I^-, and SCN^- must be present in solution to cause four-zinc insulin crystals to come out of solution. There is a question, however, as to the nature of the species in solution. Does it have the same symmetry as four-zinc insulin or could it be an even more symmetrical species capable of binding six zinc atoms?

The answer to this question may be found on close examination of the aromatic region of the NMR spectrum. The relatively simple spectrum seen in the downfield region of Figure 2a is the superposition of the lines from the four Tyr, three Phe, and two His residues which occur in each monomer. The detailed assignments, based on analogy with similar molecules, are given in Table I, where, for example, we note that the single peak in Figure 2a at 6.83 ppm is assigned to the two ortho protons on the tyrosines. Although the four tyrosines in two-zinc insulin all have different environments, they seem to produce just two peaks in Figure 2a. (The peak at 7.15 ppm is probably due to the two meta protons on Tyr.) When thiocyanate ion is added to two-zinc insulin, half of the tyrosines in the hexamer have changed their chemical shifts. This is seen in peaks 4 and 6 of the difference spectrum (Figure 2c). These are peaks which have shifted upon the addition of thiocyanate to two-zinc insulin. They are of equal area and 0.3 ppm apart, as would be expected for the protons on tyr-

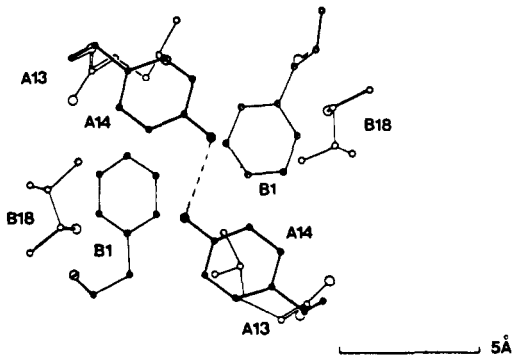

FIGURE 4: A view down the twofold axis of two-zinc insulin showing the arrangement of the B1 phenylalanines and the A14 tyrosines.

osine. The area of these two peaks totals eight protons (if we consider the area of the entire aromatic region to be 35 protons), and thus we conclude that half of the tyrosines in the hexamer have undergone a chemical-shift change. There are various ways of interpreting this fact. It seems unlikely, considering the relative mobility of portions of the insulin molecule (in particular the first eight residues of the B chain), that we are observing the NMR spectrum in solution of four-zinc insulin which has the same conformation as that reported for the crystal. The simplest way to explain the observed change would be to say that half of the tyrosines in every monomer molecule have new environments and hence new chemical shifts.

According to the X-ray crystal structure of four-zinc insulin (Bentley et al., 1976), the first eight residues of the B chain move so that the B5 histidine is close to the B10 histidine residue of a neighboring molecule (Figure 4). When this change occurs, the B1 phenylalanine of molecule I moves completely away from close contact with the A14 tyrosine of both molecules I and II. The side chain of B1 Phe, molecule II, becomes more buried and also moves out of close contact with both A14's. The arrangement of these residues in two-zinc insulin is seen in Figure 4. B26 tyrosine is on the outside of the monomer but buried in the dimer and hexamer and should not be affected greatly by the change in the first eight residues of the B chain.

From a study of molecular models of the two-zinc and four-zinc insulin structures, it seems most likely that the NMR difference spectrum can be explained by the new environments of Tyr-A19 (which was near Phe-B25 in two-zinc insulin) and the new environment of Tyr-A14 (which was near Phe-B1 in two-zinc insulin). The crystal structures of two-zinc and four-zinc insulin reveal that (within 0.5 Å) the environments of Tyr-B16 and -B26 are very similar. It is possible, of course, that addition of thiocyanate to two-zinc insulin may cause canceling effects in the chemical shifts of one or more of these tyrosines, so our observation that half of the tyrosines have new chemical shifts must be considered a lower limit. If half of the tyrosines in the hexamer have new chemical shifts and these tyrosines are always the same two in every monomer, it follows that in solution the insulin hexamer must have a twofold symmetry axis in addition to the axial threefold symmetry axis. This form of insulin should, theoretically, be capable of complexing six zinc ions.

Further interpretations of the difference spectrum are more tenuous. Although the change in peaks 7 and 8 is clear and each peak corresponds to four protons, it is not certain whether the observed changes arise from half of the β-CH_2 protons of the four tyrosines, from four β-CH_2 protons associated with the A7–B7 disulfide link which adopts a new conformation upon the addition of SCN^-, or from a combination of these

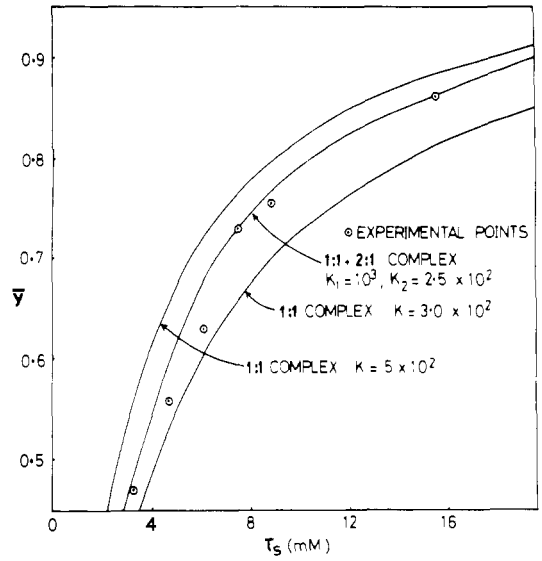

FIGURE 5: Fractional saturation (degree of binding), $\bar{y}$, vs. total SCN^- concentration, T_s (mM). Calculated curves from top to bottom are for a 1:1 complex of insulin (1.5 mM of hexamer) with SCN^- ($K = 5 \times 10^2\ M^{-1}$), a 1:1 + 2:1 complex ($K_1 = 10^3$, $K_2 = 2.5 \times 10^2\ M^{-1}$), and a 1:1 complex with $K = 3 \times 10^2\ M^{-1}$. Experimental points are from difference spectra.

two effects. The largest upfield peak in two-zinc insulin comes from the seventy-two methyl protons from the isoleucine, leucines, and valines. On addition of thiocyanate many of these reasonances shift downfield, as seen in the difference spectrum. Peak 14 in Figure 2c has an area of twenty-two protons, indicating that at least that many protons have become less shielded upon the addition of anions. In a general way this is reasonable in view of the generally more "open" structure of the anion-perturbed region of insulin compared with the more tightly packed structure of two-zinc insulin.

Titration of Two-Zinc Insulin with SCN⁻. Titration of two-zinc insulin with thiocyanate and subtraction of the subsequent spectra from the spectrum of pure two-zinc insulin give rise to a series of difference spectra like that of Figure 2c. The peak heights at a given chemical shift as a function of added thiocyanate are a measure of the binding of SCN^- to the insulin hexamer. We have considered three models[1] for this binding: a complex of 1 mol of SCN^- with 1 mol of hexamer (1:1 complex), a complex of 2 mol of SCN^- with 1 mol of hexamer (2:1 complex), and a sequential combination of these two (1:1 + 2:1 complex). In Figure 5 the curves (total thiocyanate vs. $\bar{y}$, the degree of complex formation) calculated for a 1:1 complex with two different binding constants ($K = 5 \times 10^2$ and $3 \times 10^2\ M^{-1}$) are shown as well as the curve for the sequential 1:1 + 2:1 complex ($K_1 = 10^3$, $K_2 = 2.5 \times 10^2\ M^{-1}$). The 2:1 complex can be ruled out by inspection since the calculated dependence on thiocyanate is not this strong. In Figure 5 it is seen that the experimental data (peak heights from difference spectra) cannot be made to fit a 1:1 binding curve over its entire length, but the data do fit a sequential 1:1 + 2:1 complex curve in which it is assumed that the insulin has two *equivalent* and *independent* binding sites. From the statistical nature of this type of binding, the ratio K_1/K_2 must be 4:1. This quantitative finding of anion binding at two sites on the insulin hexamer, in conjunction with the X-ray crystallographic analysis and the qualitative conclusions drawn from the NMR spectra, provides, we believe, good evidence for a symmetrical form of the insulin hexamer in solution at

[1] See Hess & Szabo (1979) for complete details of this type of analysis.

high anion concentrations, a form which might possibly be crystallized from solution, would retain six zinc ions, and would have a totally symmetric structure.

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Kinetics of Ca^{2+} Carrier in Rat Liver Mitochondria†

Marco Bragadin, Tullio Pozzan, and Giovanni Felice Azzone*

ABSTRACT: The rate of aerobic Ca^{2+} transport is limited by the rate of the H^+ pump rather than by the Ca^{2+} carrier. The kinetics of the Ca^{2+} carrier has therefore been studied by using the K^+ diffusion potential as the driving force. The apparent V_{max} of the Ca^{2+} carrier is, at 20 °C, about 900 nmol (mg of protein)$^{-1}$ min^{-1}, more than twice the rate of the H^+ pump. The apparent V_{max} is depressed by Mg^{2+} and Li^+. This supports the view that the electrolytes act as noncompetitive inhibitors of the Ca^{2+} carrier. The degree of sigmoidicity of the kinetics of Ca^{2+} transport increases with the lowering of the temperature and proportionally with the concentration of impermeant electrolytes such as Mg^{2+} and Li^+ but not choline. The effects of temperature and of electrolyte do not support the view that the sigmoidicity is due to modifications of the surface potential. Rather, they suggest that Ca^{2+} transport occurs through a multisubunit carrier, where cooperative phenomena are the result of ligand-induced conformational changes due to the interaction of several allosteric effectors with the carrier subunits. In contrast with La^{3+} which acts as a competitive inhibitor, Ruthenium Red affects the kinetics by inducing phenomena both of positive and of negative cooperativity. The Ruthenium Red induced kinetics has been reproduced through curve-fitting procedures by applying the Koshland sequential interaction hypothesis to a four-subunit Ca^{2+} carrier model.

Mammalian cells possess two main systems to control Ca^{2+} concentration in the cytosol: one, the Ca^{2+}–ATPases of the plasma membrane and endoplasmic reticulum; two, the H^+ pump of mitochondria driving an electrical Ca^{2+} carrier. However, the precise mechanism by which these two transporting systems regulate the Ca^{2+} concentration in the living cell is not understood. In heart Affolter et al. (1976) and Carafoli (1975) have proposed a role of mitochondria in regulating myocardial contraction. In the axoplasm, Brinley et al. (1977) have reported that mitochondria buffer the Ca^{2+}

† From the C.N.R. Unit for the Study of Physiology of Mitochondria and Institute of General Pathology, University of Padova, 35100 Padova, Italy. *Received May 22, 1979.*

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