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Isoelectric focusing studies of concanavalin A and the lentil lectin

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SUMMARY

Isoelectric focusing (IEF) of metallized and demetallized preparations of concanavalin A (Con A) consisting of either intact or fragmented subunits shows different band patterns. Metallized Con A consisting of intact polypeptide chains (intact Con A) has an isoelectric point (p*I*) 8.35. Metallized preparations consisting of fragmented chains (fragmented Con A) show three bands with p*I* values 8.0, 7.8 and 7.7. Demetallized intact Con A (intact apoCon A) has a p*I* of 6.5, however, it undergoes pH dependent association during IEF under certain conditions, which gives rise to multiple bands. Ampholyte-mediated demetallization of intact and fragmented Con A and subsequent aggregation of the apoprotein results in multiple bands during IEF in the presence of the pH range 3 to 10 ampholytes. However, ampholytes of the pH range 7 to 9 do not demetallize the proteins and show a single band with intact Con A. The p*I* of intact Con A remains essentially the same in the presence of inhibitory sugar. Furthermore, different moleculars forms of Con A, including locked and unlocked conformers of intact apoCon A, and the dimeric and tetramic states of both intact Con A and intact apoCon A have been identified and their p*I* values determined.

IEF of the lentil isoelectins, LcH-A and LcH-B, shows single bands of p*I* 8.5 and 9.0, respectively. However, the native lectin mixture gives rise to an additional band of p*I* 8.8 due to a hybrid protein formed by ampholyte-mediated subunit exchange between the isolectins.

INTRODUCTION

Concanavalin A (Con A), the D-glucose/D-mannose-specific lectin from jackbean seeds[1], has found wide application in biological studies[2,3]. It is a metalloprotein containing Mn^{2+} and Ca^{2+} (ref. 1). The native lectin contains intact polypeptide chains of molecular mass (M_r) 26 000 daltons, and "fragmented" chains of molecular masses 13 000, 11 000 and 10 600 daltons[4–6]. The fragmented chains appear to result from the posttranslation cleavage of a precursor polypeptide chain and the intact

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polypeptide chain is formed by subsequent transpeptidation and reannealing of the two fragments[7].

Controversy still exists in the literature concerning the isoelectric focusing (IEF) pattern of the protein. Entlicher *et al.*[8] reported isoelectric points (p*I*) from 4.5 to 5.5, while others[9–12] found p*I* values from 6.0 to 8.0. In addition, Con A shows multiple bands on IEF which some authors concluded is probably due to the presence of several molecular species formed by different combinations of fragmented and intact subunits[8–10], while others assert it is due to different conformational states of the protein[11].

There are many reports in the literature of artifacts associated with the IEF of proteins (*cf.* ref. 13). These include chelation of metal ions of metalloproteins and metalloenzymes by ampholytes[14], ampholyte binding to proteins[15], and ampholyte mediated subunit exchange[16,17]. In the present study, we demonstrate that the multiple band patterns in IEF of homogeneous, metallized preparations of Con A are due to chelation of the metal ions by ampholytes and consequent pH dependent aggregation of the apoprotein. However, under appropriate conditions intact Con A shows a single band of pI 8.35. We also report pI values of different molecular forms of Con A, including the two conformational states of intact apoCon A —the "locked" and "unlocked" conformers[18]— and the dimeric and tetrameric states of the protein.

We also report IEF studies of the lentil lectin (LcH) which possesses similar monosaccharide specificity as Con A[1]. The native lectin consists of equal amounts of two isolectins, LcH -A and LcH-B[1], which individually show single bands with characteristic p*I* values. The native mixture, however, shows an additional band due to the formation of a hybrid protein in the presence of ampholytes.

MATERIALS AND METHODS

Native Con A was purchased from Miles-Yeda (Rehovot, Israel). LcH native mixture and the isolectins, LcH-A and LcH-B, were purified from locally purchased seeds (*Lens culinaris* sub. *Macrosperma*) as described[19,20]. Ampholyte solutions of different pH ranges were obtained from Pharmacia LKB (Piscataway, NJ, U.S.A.) and Serva Fine Biochemicals (Heidelberg, F.R.G.). Monosaccharides were products of Sigma (St. Louis, MO, U.S.A.) and Pfanstiehl Laboratories (Waukegan, IL, U.S.A.).

Preparation of intact and fragmented Con A

This was done by modification of a previously described procedure[21]. About 250 mg of native Con A was dissolved in 20 m*M* Tris–HCl buffer, pH 7.2, containing 0.1 *M* NaCl, 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$ at about 5 mg/ml. The solution was allowed to stand for 30 min, clarified by centrifugation and applied to a Sephadex G-75 column (40 × 2.6 cm) equilibrated at room temperature in 20 m*M* Tris–HCl buffer, pH 7.2, containing 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$. The column was eluted with 20 m*M* glucose in the equilibrating buffer at a flow of 8–10 ml/h until the absorbance went down to below 0.1 (about 600 ml). Fractions having absorbance at 280 nm greater than 0.3 were pooled and rechromatographed to get fragmented Con A. Con A enriched in intact subunits was eluted at 20 ml/h with 0.1 *M* glucose in the equilibrating buffer. This fraction was rechromatographed to get intact Con A. The preparations were then dialyzed against water and stored as salt-free lyophilizates.

Preparation of intact apoCon A

This was done by demetallization of intact Con A as described[22].

Preparation of "locked" intact apoCon A

Intact apoCon A in the "locked" conformation was prepared by dissolving the protein in pH 5.3 or 6.4 buffer (10 m*M* sodium acetate, 0.1 *M* NaCl) containing 1 *M* α-MM (methyl α-D-mannopyranoside) and 100 μ*M* EDTA, and allowing the solution to stand for 6 days at room temperature[23].

Preparation of sample for IEF

Metallized Con A samples (native, intact or fragmented) were dissolved in pH 5.3 (10 m*M* sodium acetate, 0.1 *M* NaCl) or 7.2 (10 m*M* Tris–HCl, 0.1 *M* NaCl) buffers each containing 2 m*M* $MnCl_2$ and 2 m*M* $CaCl_2$ at about 10 mg/ml[18]. Solutions at pH 5.3 were allowed to stand overnight at 4°C and diluted 10-fold with ice-cold 10% glycerol immediately before the IEF run. Solutions at pH 7.2 were allowed to stand overnight at 25°C and diluted similarly with the glycerol solution at the same temperature. Intact apoCon A was treated in the same manner except that pH 7.2 buffer was replaced by pH 6.4 buffer (10 m*M* sodium acetate, 0.1 *M* NaCl) to avoid precipitation of the apoprotein[24], and $MnCl_2$ and $CaCl_2$ were replaced by 100 μ*M* EDTA in the buffer.

Isoelectric focusing

IEF was done according to Wrigley[25] using 7.5% gel and 2% ampholyte solutions. For runs in the presence of monosaccharides, the sugar is included in the gel solutions at the appropriate concentrations. Unless stated otherwise, the runs were made at 4°C with the cathode at the top (catholyte: 0.08% NaOH)[26] and anode at the bottom (anolyte: 0.2% H_2SO_4). The bands were stained with 1% Coomassie Blue and their relative intensities measured by a Joyce Loebl Chromoscane 3 gel scanner. The pH gradient was determined by eluting 5 mm thick gel slices with 1 ml CO_2-free glass-distilled water[26]. Whenever possible, complete focusing was ensured by simultaneously running a mixture of cow α-lactoglobulins A and B (the p*I* values[27] of the A and B variants are 5.35 and 5.41, respectively). In other cases, LcH-A (pI 8.5; see later) was used.

Polyacrylamide gel electrophoresis (PAGE)

This was done in alkaline pH essentially as described[28]. The pH of the gel and the tray buffers were adjusted to appropriate values. Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was done acording to Weber and Osborn[29].

RESULTS

Preparation of intact Con A and fragmented Con A

Attempts at preparing intact Con A by the method of Cunningham *et al.*[6] which involves incubating native Con A with 1% NH_4HCO_3 did not work in our hands. Among other methods reported[4,5,21], the procedure of Williams *et al.*[21] is the simplest and the most convenient. However, the problem of the latter procedure is

relatively poor yield. Overall recovery of the protein from a Sephadex G-75 column was reported to be about 50%[21]. In our modified procedure, native Con A was dissolved and loaded on the column in a buffer containing a higher salt concentration (0.1 *M* NaCl) which stabilized the protein, and then eluted with low ionic strength buffer as described[21]. The overall recovery of the protein increased to 99%.

Elution of the adsorbed protein from the Sephadex G-75 column with 20 m*M* glucose gives a broad peak (approximately 20% of the total load) which is enriched in fragmented polypeptide chains. This fraction was rechromatographed to obtain fragmented Con A preparation which, by SDS gel electrophoresis, was found to contain about 55% of the 13 000-dalton chain and 42% of the 11 000- and 10 600-dalton chains together.

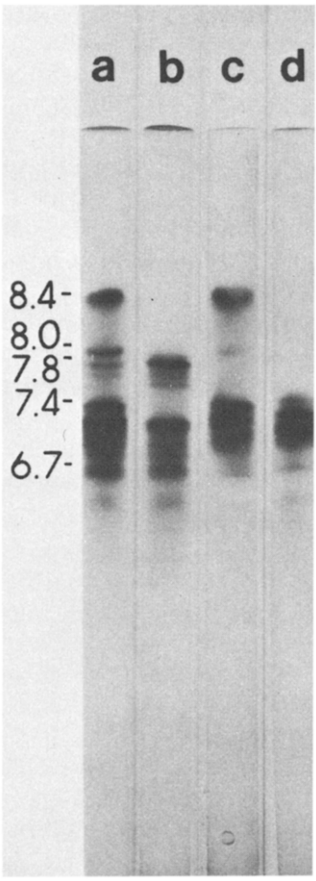
The fraction eluted from the column by 0.1 *M* glucose emerged as a sharp peak (78–80% of the total load), and is composed of approximately 80% intact chains. Rechromatography of this fraction gives a preparation of intact Con A containing about 98% intact polypeptide chain.

Flow-rate during elution with 20 m*M* glucose is an important factor. The results described above were obtained at a flow-rate of 8 ml/h. However, when the flow-rate was increased to 30 ml/h, the proportion of the second peak is reduced to 40% of the total protein, and the absorbance at 280 nm did not go below 0.25 after the first peak had emerged due to "bleeding" of the protein from the column.

IEF of native, fragmented and intact Con A

Studies were carried out with Con A preparations incubated in a pH 5.3 buffer at 4°C. Under these conditions, the protein exists essentially as a dimer[30]. Fig. 1 shows the IEF patterns of native, fragmented and intact Con A as well as intact apoCon A using ampholytes of the pH range 3 to 10. The top most band (pI 8.4) of native Con A (Fig. 1a) is also present in intact Con A (Fig. 1c). The three middle bands of native Con A at pI 8.0, 7.8 and 7.7 correspond to the three top bands of fragmented Con A (Fig. 1b). None of these bands are seen with intact apoCon A (Fig. 1d). Therefore, these bands are due to the fully metallized forms (intact or fragmented) of Con A. The multiple bands seen with native Con A between p*I* 7.4 to 6.7 (Fig. 1a) are also present in fragmented Con A (p*I* 7.2 to 6.7) (Fig. 1b), intact Con A (p*I* 7.4 to 7.0) (Fig. 1c), and intact apoCon A (p*I* 7.4 to 7.0) (Fig. 1d). Thus, these bands are clearly due to the apoproteins. Since native, fragmented and intact Con A samples were preincubated overnight in the presence of Mn^{2+} and Ca^{2+} in order to ensure fully metallized proteins[20], the apoprotein bands are due to demetallization during the run.

Fig. 2 shows the results of IEF studies with native and intact Con A, and intact apoCon A using ampholytes of the pH range 7 to 9. The samples were treated in the same way as above before the runs. Native Con A gave major bands at p*I* 8.35, 8.05 and 7.75, and a minor band at 7.60 (Fig. 2a). Intact Con A focused as essentially a single band at pI 8.35 (a trace of the pI 8.05 band is also seen with intact Con A) (Fig. 2b). Thus, the results using ampholytes of both pH ranges show that the p*I* of fully metallized intact Con A is 8.35. Intact apoCon A shows a broad band at the bottom of the gel (Fig. 2c), as expected from the results using ampholytes of the pH range 3 to 10. Neither native or intact Con A show any band in this region using ampholytes in the pH range 7 to 9, indicating the lack formation of apoproteins. Thus, the bands at



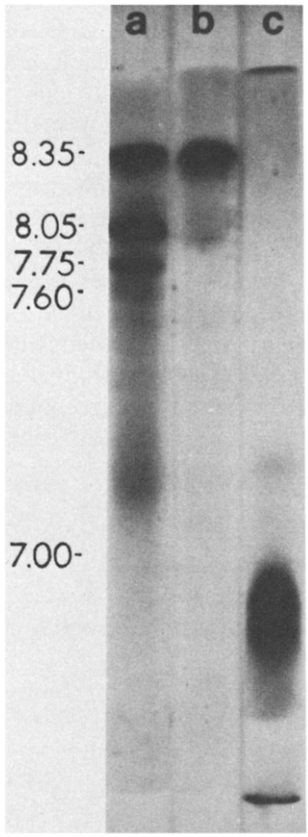


Fig. 1. IEF patterns of (a) native, (b) fragmented, and (c) intact Con A, and (d) intact apoCon A in the pH range 3 to 10. The pH decreases from the top to the bottom of the gels. The direction of migration is also from the top to the bottom. Loads: (a), 150 μg; (b) and (c), 100 μg; and (d), 50 μg.

Fig. 2. IEF patterns of (a) native and (b) intact Con A, and (c) intact apoCon A in the pH range 7 to 9. The pH decreases from the top to the bottom of the gels. The direction of migration is also from the top to the bottom. Loads: (a) and (c), 100 μg; (b), 50 μg.

pI 8.05, 7.75 and 7.6 of native Con A correspond to fully metallized fragmented Con A molecules. These p*I* values of fragmented Con A are essentially the same as those obtained by IEF in the pH range 3 to 10.

IEF in the pH range 3 to 10 in the presence of 0.1 *M* α-MM, a monosaccharide that binds to Con A[1], shows a single band of pI 8.5 with intact Con A and close multiple bands of p*I* 7.8 to 7.5 with fragmented Con A (not shown). Thus, binding to a specific monosaccharide has little effect on the pI of the protein.

Studies were also carried out with intact Con A tetramers (protein incubated in pH 7.2 buffer at 25°C [ref. 30]). IEF at 25°C using ampholytes in the pH range 7 to 9 results in a major band at p*I* 8.15, along with minor bands at the bottom of the gel, which are, therefore, associated with the apoprotein (not shown). The results show that dimeric Con A has a slightly higher p*I* than tetrameric Con A.

IEF of "unlocked" and "locked" forms of intact ApoCon A

In IEF of intact apoCon A, described above (Fig. 1d), the sample treatment results in predominantly the "unlocked" form of the protein in solution[18]. IEF of the same sample of intact apoCon A also showed multiple bands in the pH range 5 to 8 (Fig. 3a). Following reported procedures[25,26], the cathode was placed at the top and the anode at the bottom in this and previous experiments. On the other hand, IEF of the above sample in the same pH range with reversed electrode polarities (anode at the top and cathode at the bottom) showed one major band (approximately 85% of the total load) corresponding to a pI of 6.5 (Fig. 3b). Thus, p*I* of intact apoCon A dimer in the "unlocked" conformation is 6.5.

Fig. 3c shows the results of IEF with anode at the top and cathode at the bottom in the pH range 5 to 8 of intact apoCon A in the "locked" from prepared by incubating the protein with 1 *M* α-MM in pH 5.3 buffer at 4°C (ref. 23). Two major bands of p*I* 6.25 and 6.4 are observed, along with a minor band of pI 6.5. The pI of the latter band corresponds to the "unlocked" form of the apoprotein. Thus, the two major bands appear to be related to the apoprotein in the "locked" conformation.

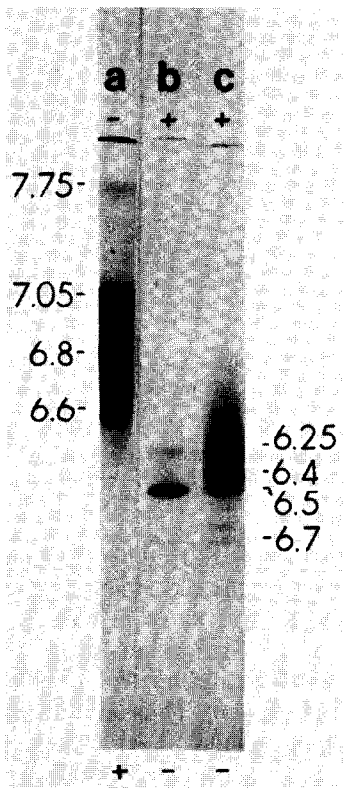


Fig. 3. IEF patterns of intact apoCon A in the pH range 5 to 8 in (a,b) "unlocked" and (c) "locked" conformations. The pH decreases from the top to the bottom in (a), and increases in (b) and (c). The direction of migration is from the top to the bottom for all gels. Gel (c) was run in the presence of 1 *M* α-MM. Loads: (a) and (c), 100 μg; (b), 35 μg.

The intensities of the major bands are approximately equal. These bands are also observed as minor species in Fig. 3b, which is consistent with the presence of smaller amount of the "locked" form under the condition of sample incubation[18]. The presence of two locked apoCon A bands, however, required further experimental evidence to suggest their identity.

"Locked" apoCon A prepared by incubation in 1 *M* α-MM in pH 6.4 buffer at 25°C also showed two major bands of p*I* 6.25 and 6.4 on IEF at 25°C (anode at the top and cathode at the bottom, however, the ratio of the intensities of the bands was approximately 70:30 (not shown). Since Con A tetramer is predominant over the dimer at higher temperature and pH[30], the present results suggest that the p*I* 6.25 band corresponds to the apoprotein tetramer in the "locked" conformation, and the pI 6.4 band corresponds to the dimer.

IEF of LcH

Fig. 4 shows the results of IEF of LcH native mixture and the two isolectins, LcH-A and LcH-B, using ampholytes of the pH range 3 to 10. The patterns of the

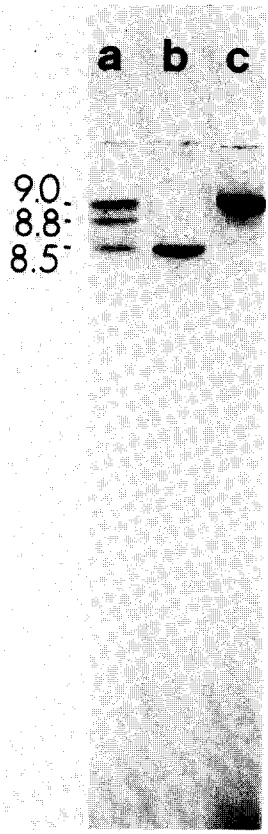


Fig. 4. IEF patterns of (a) LcH native mixture, (b) LcH-A, and (c) LcH-B in the pH range 3 to 10. The pH decreases from top to bottom of the gels. The direction of migration is also from the top to the bottom. Loads: (a), 50 μg; (b) and (c), 30 μg.

native mixture (Fig. 4a) and an equimolar mixture of the two isolectins are similar, both giving three bands, two of which correspond to the two isolectins of p*I* (LcH-A) and 9.0 (LcH-B) (Fig. 4b and c, respectively). The third band which constitutes approximately 25% of the total load appears in the middle and has a p*I* of 8.8. When the middle band is isolated and refocused, it shows a pattern similar to that of the native mixture. IEF of LcH native mixture in the pH range 7 to 10 shows a similar pattern except that the middle band constitutes about 10% of the total load. IEF of the native mixture in the presence of 0.1 *M* α-MM using ampholytes of the pH range 3 to 10 shows the same pattern as in the absence of sugar with no change in p*I* values.

Fig. 5 shows the results of PAGE of LcH native mixture and the two isolectins at pH 9.4. The native mixture (lane c) shows two sharp bands corresponding to LcH-A (lane a) and LcH-B (lane b). Preincubation of the native mixture with 1% ampholyte solution of the pH range 3 to 10 gives the same pattern (lane d) as the native mixture in the absence of ampholytes. Furthermore, the protein of p*I* 8.8 also shows two bands corresponding to LcH-A and LcH-B on PAGE (not shown).

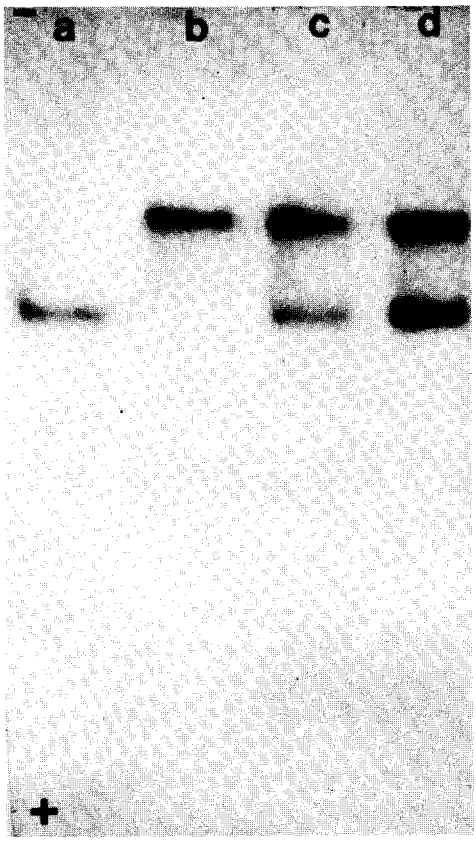


Fig. 5. Polyacrylamide gel electrophoresis at pH 9.4 of LcH-A (lane a, 30 μg), LcH-B (lane b, 30 μg), LcH native mixture (lane c, 60 μg) and LcH native mixture preincubated with 1% ampholyte solution of the pH range 3 to 10 (lane d, 100 μg). The direction of migration is from the top to the bottom of the gel.

DISCUSSION

IEF studies of Con A

The present results show that IEF techniques can be used to characterize not only the fully metallized intact and fragmented Con A but also different molecular forms of the lectin, including the apoprotein, the "locked" and "unlocked" conformers, and the dimeric and the tetrameric states of the protein.

Fig. 1 and 2 show that intact Con A can be focused as a single band, with a p*I* of 8.35 in the presence of ampholytes of pH ranges 3 to 10 and 7 to 9, respectively. Candiano *et al.*[10] recently reported a p*I* of 7.65 for intact Con A using ampholytes of the pH range 7 to 11. However, under these conditions the protein is exposed to a pH above 9 (ref. 31), and undergoes irreversible denaturation[32] with concomitant loss of metal ions[24] to generate apoCon A.

Fragmented Con A shows three major bands in either pH range (excluding the additional bands in the pH range 3 to 10) at p*I* values 8.0, 7.8 and 7.7. SDS-PAGE of fragmented Con A showed that the intact chain was essentially absent in the preparation. Thus, it appears that these bands are due to proteins formed by different combinations of the M_r 13 000-, 11 000- and 10 600-dalton polypeptide fragments.

The pattern of the native Con A appears to be a combination of intact and fragmented Con A (Figs. 1 and 2). The lack of any additional band in native Con A suggests the absence of hybrid protein molecules consisting of both intact and fragmented polypeptide chains.

Native and intact Con A show different IEF band patterns using ampholytes of the pH ranges 3 to 10 and 7 to 9 (Figs. 1 and 2, respectively). Although the metallized proteins are focused essentially at the same pH in the presence of either carrier ampholytes, both protein samples show multiple bands due to formation of apoproteins in the presence of ampholytes of the pH range 3 to 10 but not 7 to 9 range. Since both protein samples were preincubated with Mn^{2+} and Ca^{2+} to ensure full metallization[18], the results demonstrate that formation of apoproteins was mediated by ampholytes specific for the pH 3 to 10 range but not the pH 7 to 9 range. The latter results also show that demetallization of the protein during IEF is not mediated by the applied electric gradient.

IEF of intact and fragmented Con A in the presence of α-MM with ampholytes of the pH range 3 to 10 showed that the specific binding sugar prevents the formation of apoproteins. These results are consistent with tighter binding of the metal ions to the protein in the presence of bound monosaccharides[18]. The results also showed that the p*I* values of intact and fragmented Con A do not change due to monosaccharide binding, although the binding involves a conformational change[32] and masking of titrable carboxyl groups[33] of Con A. The results contradict the conclusion of Akedo *et al.*[9] that the p*I* values of the protein undergo extensive shifts toward alkaline pH due to monosaccharide binding. These authors found essentially the same IEF patterns as the present report using ampholytes of the pH range 3 to 10 in the presence and absence of sugar, however, the bands below pH 7.4 in the absence of sugar were due to the formation of apoprotein.

Con A exists in dimer–tetramer equilibrium, with the dimer predominating below pH 5.6 and at low temperatures, and the tetramer above pH 7.0 and at room temperature[30]. The results with intact Con A and intact apoCon A show that the

dimers have slightly higher p*I* values (by about 0.2 pH units) than the corresponding tetramers. Differences in the p*I* values between the dimeric and tetrameric states of Con A are expected in view of the involvement of Lys 114 and 116, Glu 192, His 51 and 121, and Ser 108 and 117 residues in the dimer–dimer contact region[34].

The results in Figs. 1 and 2 show that intact apoCon A gives multiple bands in the pH range 3 to 10, though the corresponding metallized protein gives a single band. This is not due to cleavage of some of the amide linkages of Con A during demetallization by 0.1 *M* HCl, since SDS-PAGE of intact Con A and intact apoCon A show single bands of M_r 26 000 daltons. There may be two other possibilities. The multiple bands may be formed either due to aggregation of intact apoCon A, since it undergoes association at a pH greater than 6.5 (ref. 24), or due to ampholyte binding to intact apoCon A, as has been observed with certain other proteins[13,15]. To test these possibilities, we performed IEF using ampholytes of the pH range 5 to 8, first placing the cathode at the top of the gel and then reversing the electrode polarities.

In the former run (cathode at the top), pH decreases from the top to the bottom in the gels[13,31]. With pH 5 to 8 range ampholytes, protein entering the gel from the top experiences a pH higher than 6.5 (the pH of 2% ampholyte solution is 6.5), since the ampholyte molecules have higher mobilities than the protein in polyacrylamide gels due to the smaller size of the former[13,31]. Since apoCon A aggregates above pH 6.5, the multiple bands in Fig. 3a appear to be due to aggregation of apoCon A. In the latter run (anode at the top) (Fig. 3b), the pH increases from the top to the bottom of the gels, and the protein experiences a pH lower than 6.5, as it enters the gel. Thus, the results indicate that the multiple bands of intact apoCon A are due to the pH-dependent aggregation of the apoprotein. Had the multiplicity been due to ampholyte binding to the protein, the same, but inverted pattern is expected by the exchange of electrode polarity.

Con A exists as an equilibrium mixture of two conformational states. The "unlocked" state is the predominant conformation (87%) of apoCon A at pH 6.4 and 25°C, which weakly binds both metal ions and saccharides[18]. (The results in Fig. 3b show essentially the same percentage for the major protein band in the "unlocked" apoCon A preparation.) The "locked" conformation tightly binds two metal ions per monomer and, once fully metallized, possesses full saccharide binding activity[18]. Addition of specific sugar to apoCon A solution shifts the overall equilibrium toward the "locked" conformation due to the higher sugar binding activity of the latter[23]. The present findings (Fig. 3b and c) show that IEF techniques can distinguish the p*I* values of the two conformers of intact apoCon A. (Similar studies with the metallized protein are not possible. Addition of metal ions to apoCon A quickly converts the "unlocked" protein to the "locked" conformation[18].) The results also show that intact apoCon A in the "locked" conformation, prepared in the presence of the inhibitory saccharide, exists as a temperature-dependent mixture of dimer and tetramer (Fig. 3c). However, at present we do not know whether the dimer-tetramer equilibrium of "locked" apoCon A is an intrinsic property of the protein, or if the bound saccharide influences this equilibrium.

IEF studies of LcH

Native LcH which consists of two isolectins shows three bands upon IEF, two of which correspond to the two isolectins at p*I* values 9.0 and 8.5 (Fig. 4). The third

band at p*I* 8.8 when isolated gives the same pattern as the native mixture on IEF, and two bands corresponding to the two native isolectins on PAGE. Thus, the third band appears to be a hybrid of the two isolectins formed by subunit exchange between the two isolectins. Furthermore, the native mixture preincubated with 1% ampholyte shows two bands on PAGE corresponding to the two isolectins. Since ampholyte molecules are separated from the protein during PAGE due to their faster mobilities[13,31], the results indicate that the presence of ampholytes is required for the formation and stability of the hybrid band, and that upon removal of the ampholytes dissociation of the hybrid protein to the native isolectins is rapid. Interestingly, the proportion of the hybrid band depends on ampholyte composition, since the intensity of the band is different between the pH 3 to 10 and 7 to 10 IEF runs. Formation of a hybrid protein by ampholyte-mediated subunit exchange between the isolectins has also been reported for the pea lectin[17], which is closely related to LcH and consists of two isolectins[1]. However, contrary to the observation with pea lectin[17], the hybrid LcH is not produced by the prolonged exposure of the protein to alkaline pH.

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