Determination of Chemical Properties of Individual Histidine and Tyrosine Residues of Concanavalin A by Competitive Labeling with 1-Fluoro-2,4-dinitrobenzene†

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ABSTRACT: A selective peptide-mapping procedure was devised to purify peptides containing histidine or tyrosine residues from proteolytic digests of concanavalin A (Con A). The protein was modified with maleic anhydride followed by 1-fluoro-2,4-dinitrobenzene (Dnp-F) and then digested with thermolysin. The resulting labeled peptides were separated by high-performance liquid chromatography, and the Dnp-histidine and Dnp-tyrosine peptides were identified by their spectral characteristics. From their amino acid compositions, the labeled peptides could all be assigned within the known sequence. Peptides representing five of the six histidines and all seven tyrosines were obtained. With the same peptide-mapping procedure, the chemical properties (pK and reactivity) of these residues were determined. Samples of concanavalin A at various pH values were labeled with trace amounts of [^{3}H]Dnp-F, in the presence of Gln-Gly as an internal standard. To each sample was added an aliquot of a mixture of [^{14}C]Dnp-Gln-Gly and [^{14}C]Dnp-maleyl-Con A. Portions of each sample were removed, [^{14}C]Dnp-Ala-Ala and ϵ-[^{14}C]Dnp-lysine were added, and the mixtures were hydrolyzed. The various Dnp amino acid derivatives were purified by HPLC. The remainder of each [^{3}H]Dnp sample was maleylated, dinitrophenylated, and digested with thermolysin and separated by HPLC as above. From the ^{3}H/^{14}C ratios of the Dnp amino acid derivatives and the Dnp peptides relative to the ratio of the internal standard, pK and reactivity data were obtained for (a) the average behavior of the lysine, histidine, and tyrosine residues and (b) the individual behavior of the N-terminal alanine residue and the five histidine and seven tyrosine residues in the protein. The properties of these residues are discussed with respect to their functional roles and their local environments within the protein structure.

Chemical methods for identifying and characterizing residues that form interaction sites on proteins have largely focused on residues in the active sites of enzymes. Here, advantage can be taken of the unique chemistry of the catalytic residues or of the enzyme's specificity to devise affinity or other labels. It is more difficult to study residues in other types of site, particularly those involved in protein–protein interactions such as subunit interfaces and antigenic determinants. One general method is competitive labeling (Kaplan et al., 1971) or differential chemical modification (Bosshard, 1979), which allows the chemical properties of individual residues of a given type to be determined. By comparison of these properties in the presence and absence of interacting ligand, key residues in the site may be identified. An example of this approach is the location of the lysine residues in cytochrome *c* that interact with cytochrome *c* oxidase, by means of acetylation with [^{3}H]acetic anhydride (Rieder & Bosshard, 1980).

Histidine and tyrosine residues often occur in interaction sites. Participation of tyrosine residues may be followed by UV or CD[1] difference spectroscopy and of histidine residues by NMR, but identification of the individual residue associated with particular spectroscopic properties is difficult when several are present in a protein. We describe a competitive labeling procedure that both determines the reactivity and pK values of individual histidine and tyrosine residues and allows their assignment within the protein sequence. The protein studied was concanavalin A in which two tyrosine residues are known to form part of the carbohydrate binding site (Hardman, 1979), one histidine residue occurs at the double metal binding site (Hardman et al., 1982) in the subsite for transition metals, and others are at the various monomer–monomer interfaces in the protein tetramer (Reeke et al., 1975). The reagents used were [^{3}H]- and [^{14}C]-1-fluoro-2,4-dinitrobenzenes, which have previously been used to determine the average properties of the histidine, tyrosine, lysine, cysteine, and *N*-terminal residues of immunoglobulins (Kaplan et al., 1980) and of individual residues in glucagon (Cockle et al., 1982). The spectroscopic properties of the Dnp derivatives make this reagent particularly suitable for high-performance liquid chromatography of derivatized peptides.

Materials and Methods

Con A was purified by the method of Agrawal and Goldstein (1967). [^{3}H]Dnp-F, sp act. 16.6 Ci/mmol, [^{14}C]Dnp-F, sp act. 12 mCi/mmol, and ACS scintillation fluid were obtained from Amersham Canada Ltd. HPLC columns (0.46 × 25 cm) were packed with Spherisorb ODS-2, 5 μm (Phase Sep), in a Shandon HPLC column packing instrument. *O*-Dnp-tyrosine, ϵ-Dnp-lysine, and Ala-Ala were from Sigma. Gln-Gly was from Vega Biochemicals. ϵ-[^{14}C]Dnp-lysine was a gift from Dr. H. Kaplan.

Peptide Mapping of Fully Modified Con A. Con A was fully maleylated on the N-terminal and lysine amino groups (Young & Leon, 1974) by treating the protein (10 mg) in 50

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[1] Abbreviations: CD, circular dichroism; Con A, concanavalin A; Dnp, 2,4-dinitrophenyl; Dnp-F, 1-fluoro-2,4-dinitrobenzene; HPLC, high-performance liquid chromatography.

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mM barbital buffer, pH 8.6 (2 mL), with maleic anhydride (10 mg). The pH was maintained at 8.6 by addition of 1 N NaOH with a Radiometer pH stat. After the reaction was complete, $NaHCO_3$ (0.5 g) was added. The tyrosine and histidine residues were then fully dinitrophenylated. The maleyl-Con A was made 8 M with respect to urea, Dnp-F (100 μL, 50% v/v in acetonitrile) was added, and the reaction was allowed to proceed with vigorous mixing for 18 h at room temperature, protected from light. The labeled protein was dialyzed vs. 2 × 8 L of distilled water followed by 2 × 8 L of 1% NH_4HCO_3. The Dnp-maleyl-Con A was digested with thermolysin (400 μg) for 5 h at 37 °C and then lyophilized.

HPLC separations of the Dnp-labeled peptides (5 mg of digest) were carried out with a 5-μm Spherisorb ODS-2 column (0.46 × 25 cm). The HPLC equipment was from Beckman Instruments, and the eluate was monitored simultaneously at 320 and 270 nm with a Beckman Model 165 variable-wavelength detector. Fractions of 0.6 mL were collected in polypropylene plates on a Gilson FC80 collector. Initial separations were run in 10 mM sodium phosphate, pH 7.4, with a gradient of acetonitrile from 0 to 20% at 0.25%/min and from 20 to 40% at 0.4%/min. Fractions corresponding to the major peaks were pooled, lyophilized, redissolved in 0.5 mL of 10 mM ammonium acetate, pH 5.3, and recycled in this buffer and a gradient of 0.5%/min of acetonitrile containing 0.05% acetic acid.

The peak fractions were pooled and identified as histidine- or tyrosine-containing peptides on the basis of the A_{270nm}:A_{320nm} ratio. This ratio is 1.1 for *O*-Dnp-tyrosine and 2.0 for imidazolyl-Dnp-histidine (S. A. Cockle and N. M. Young, unpublished observations). The total amount, in nanomoles, of Dnp peptide present in each pool was estimated from the extinction coefficients of the Dnp amino acids. At 320 nm, the extinction coefficient is 7970 M^{-1} cm^{-1} for *O*-Dnp-tyrosine and 5400 M^{-1} cm^{-1} for imidazolyl-Dnp-histidine. After lyophilization, the peptides were hydrolyzed in 0.5 mL of 6 N HCl for 22 h at 110 °C in sealed, evacuated tubes. The hydrolysates were dried in a vacuum desiccator over NaOH pellets. Amino acid analyses were performed on a Durrum D500 analyzer.

Preparation of [^{14}C]*Dinitrophenyl Derivatives.* Con A (4.6 mg/mL, 35 mg total) in barbital buffer was treated with maleic anhydride (35 mg) as above. $NaHCO_3$ (0.5 g) was added, and the solution was made 8 M in urea. [^{14}C]Dnp-F (60 μCi, 12 mCi/mmol) was diluted 1:50 with cold Dnp-F in 0.5 mL of acetonitrile and added to the maleylated protein. The sample was stirred for 24 h, protected from light. The [^{14}C]Dnp-maleyl-Con A was dialyzed against 2 × 8 L of distilled water for 24 h.

For the preparation of the [^{14}C]Dnp derivatives of Ala-Ala and Gln-Gly, each dipeptide (4 mg) was dissolved in distilled water (1 ml) and $NaHCO_3$ (0.25 g) was added. [^{14}C]Dnp-F (10 μCi, diluted 1:50 with cold Dnp-F as above) was added and stirred 40 h at room temperature, protected from light. The solutions were acidified to pH 2.0 by addition of 12 N HCl and extracted 4 or 5 times with ether. The ether extracts were dried under a stream of N_2 gas and dissolved in chloroform (5 mL) that had been shaken with 1% Na_2HPO_4. The samples were loaded on a silica gel G column (0.5 × 4 cm) and washed with the chloroform until all Dnp-OH was eluted. The [^{14}C]Dnp peptides were eluted with 80% aqueous acetone–1% glacial acetic acid and lyophilized.

[^{3}*H*]*Dnp-F Trace Labeling of Con A.* Con A (7.6 mg/mL, 30 mg) was dialyzed into a buffer comprised of 5 mM glacial acetic acid, 5 mM *N*-methylmorpholine, 5 mM sodium borate, and 150 mM NaCl, pH 7.6. Reaction mixtures containing Con A (1.5 mg, 58 nmol) and the internal standard Gln-Gly (375 nmol) in a final volume of 1.5 mL were adjusted to the desired pH with 1 N HCl or 1 N NaOH. Samples were prepared over the pH range of 5.5–10.0 at intervals of 0.3 pH unit. [^{3}H]Dnp-F (5 μCi, 16.6 Ci/mmol, in 25 μL of acetonitrile) was added to each sample and stirred for 18 h at room temperature, protected from light. A mixture of [^{14}C]Dnp-maleyl-Con A and [^{14}C]Dnp-Gln-Gly (1.5 mg of protein and 15 000 cpm internal standard in 1.1 mL) was added to each sample. An aliquot (800 μL) of each sample was removed and combined with a second mixture of ϵ-[^{14}C]Dnp-lysine (21 250 cpm) and [^{14}C]Dnp-Ala-Ala (16 700 cpm). The aliquots were made 6 N in HCl and hydrolyzed for 22 h at 110 °C and dried over NaOH pellets. Imidazolyl-Dnp-histidine, Dnp-glutamic acid, Dnp-alanine, ϵ-Dnp-lysine, and *O*-Dnp-tyrosine were purified in an isocratic system of acetonitrile–ammonium formate, pH 3.0, described previously (Cockle et al., 1982). The remainder of each sample was fully maleylated and dinitrophenylated as described above and then digested with thermolysin for 5 h at 37 °C at an enzyme:protein ratio of 1:25. The [^{3}H,^{14}C]Dnp-Con A peptides from the thermolytic digests were separated by HPLC as described above for the cold peptides. Aliquots of each fraction were removed, mixed with 5 mL of ACS scintillation fluid, and counted on a LKB Rack Beta scintillation counter, adjusting the ^{14}C and ^{3}H windows for minimum spillover. Where necessary, peptides were recycled to confirm the ^{3}H:^{14}C ratio. To obtain this ratio for Y22 and H24 residues that occur in the same peptide, the peptide was hydrolyzed, and the Dnp amino acid derivatives were separated as described above.

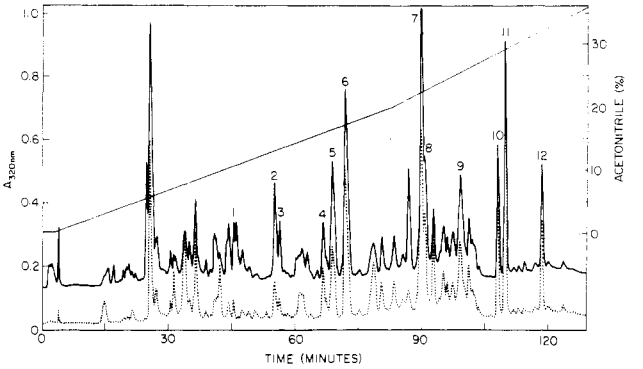

FIGURE 1: HPLC separation of Dnp-tyrosine- and Dnp-histidine-containing peptides from a thermolytic digest of Dnp-maleyl-Con A. Peptide separations were carried out in 10 mM sodium phosphate buffer, pH 7.4, with a gradient of acetonitrile as shown. The eluate was monitored simultaneously at 320 (dotted line) and 270 nm (solid line). Both wavelengths are the same sensitivity setting with the 270-nm tracing offset by 0.15 absorbance unit.

RESULTS

Peptide mapping by HPLC was carried out on proteolytic digests of various Con A derivatives to determine conditions that would give well-resolved peptides with, as far as possible, one histidine or tyrosine residue per peptide. Con A was modified with maleic anhydride to block lysine and N-terminal residues, and then the histidine and tyrosine residues were modified with Dnp-F. Use of citraconic anhydride instead of maleic anhydride and removal of the citraconyl groups prior to HPLC was also investigated. The modified proteins were reacted with thermolysin alone or chymotrypsin followed by thermolysin. The most suitable map was obtained from thermolysin digestion of Dnp-maleyl-Con A (Figure 1). Simultaneous detection of the HPLC eluate at two wavelengths

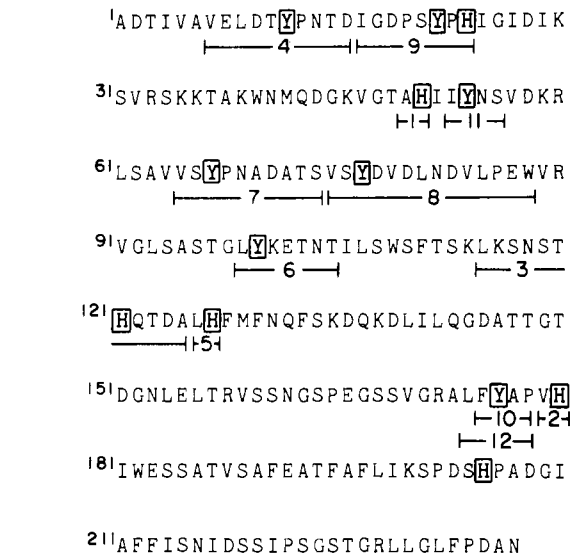

FIGURE 2: Location of tyrosine and histidine peptides within the sequence of Con A (Cunningham et al., 1975). The thermolytic peptides were identified from their amino acid compositions (Table I).

allowed assignment of the peptides to histidine or tyrosine or (histidine + tyrosine) peptides as the spectra of imidazolyl-Dnp-histidine and *O*-Dnp-tyrosine derivatives are quite distinct. The ratios, A_{270nm}:A_{320nm}, are 2.0 and 1.1 for the histidine and tyrosine derivatives, respectively (S. A. Cockle and N. M. Young, unpublished observations). The amino acid compositions of the peptides, determined after recycling (Table I), permitted assignment of the labeled peptides unequivocally to the known sequence of Con A (Figure 2). Peptides representing all seven tyrosine residues and five of the six histidine residues of Con A were identified in this way. Peptides containing H205 could not be obtained in sufficient yield, presumably due to the many potential thermolytic cleavage points in the vicinity of this residue. Only one peptide, comprising residues 17–24, contained both histidine and tyrosine residues, and none of the peptides contained multiple histidine and tyrosine residues. The sequence used here (Cunningham et al., 1975) was obtained by protein sequencing; the recently published sequence from the Con A gene (Carrington et al., 1985) does not give differences in composition for any of these peptides.

Competitive labeling of Con A at pH values between 5.5 and 10.0 was carried out by treating 1.5-mg aliquots of Con A, mixed with the dipeptide Gln-Gly as an internal standard, with [^{3}H]Dnp-F. [^{14}C]Dnp-maleyl-Con A and [^{14}C]Dnp-Gln-Gly were then added to the aliquots. A portion of each was withdrawn to be analyzed for the internal standard and for the average properties of the various residues (see below). The main samples were then fully modified with maleic anhydride followed by Dnp-F to give chemically homogeneous derivatized protein samples, which were digested with thermolysin. The labeled peptides were separated by HPLC, and their ^{3}H and ^{14}C contents were measured by liquid scintillation counting. Peptides containing both tyrosine and histidine residues were hydrolyzed, and the individual imidazolyl-Dnp-histidine and *O*-Dnp-tyrosine derivatives were purified by HPLC.

The retained portions of the labeled samples were each mixed with ε-[^{14}C]Dnp-lysine and [^{14}C]Dnp-Ala-Ala and then hydrolyzed to give Dnp-glutamic acid (from the internal standard), *O*-Dnp-tyrosine, imidazolyl-Dnp-histidine, ε-Dnp-lysine, and Dnp-alanine (from the N-terminal residue), which

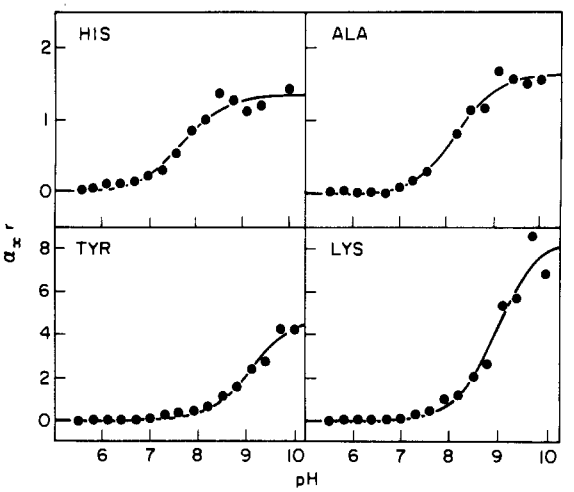

FIGURE 3: Average pH–reactivity profiles of the histidines, tyrosines, lysines, and N-terminal alanine of Con A. The lines are theoretical titration curves whose parameters are given in Table II.

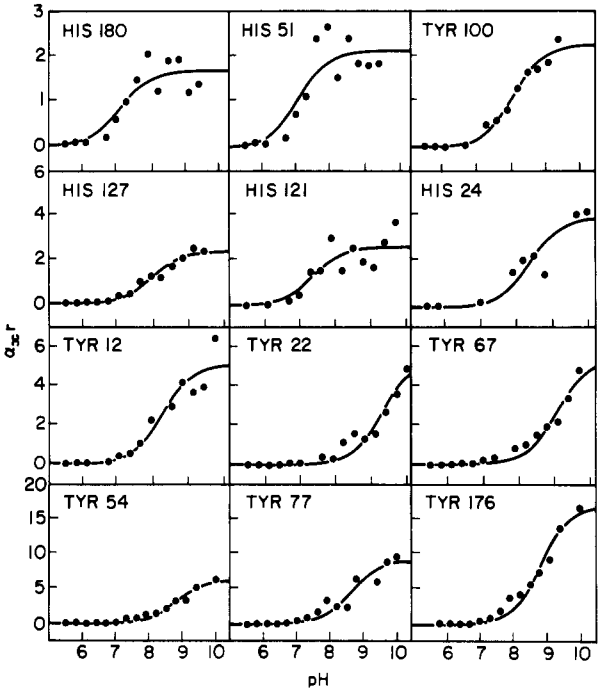

FIGURE 4: pH–reactivity profiles of individual histidine and tyrosine residues of Con A. The lines are theoretical titration curves whose parameters are given in Table II.

were again separated by HPLC, and their ^{3}H and ^{14}C contents were measured.

The data were analyzed with the expression (Kaplan et al., 1971):

$$\alpha_x r = \alpha_s(^3H/^{14}C)_x/(^3H/^{14}C)_s$$

Here, α_x is the degree of ionization of the functional group under study, α_s is the degree of ionization of the internal standard (pK = 7.63), r is the pH-independent second-order velocity constant for the reaction of the functional group under study relative to that of the standard, and $(^3H/^{14}C)_x$ and $(^3H/^{14}C)_s$ are the radioactivity ratios for the group and the internal standard, respectively. Theoretical titration curves were fitted to the data by a nonlinear least-squares procedure. The data for the average behavior of the lysine, tyrosine, and histidine side chains and N-terminal alanine are shown in Figure 3 and for the individual tyrosine and histidine residues are shown in Figure 4. The pK values and reactivities for the

Table I: Amino Acid Compositions and Sequence Assignments of Labeled Histidine and Tyrosine Peptides[a]

	peptide no.											
amino acid	1	2	3	4	5	6	7	8	9	10	11	12
Asx			2.01 (2)	2.60 (3)		1.29 (1)	2.01 (2)	3.79 (4)	1.53 (1)	0.20 (0)	0.97 (1)	
Thr		0.21 (0)	1.69 (2)	1.67 (2)		2.07 (2)	0.81 (1)	0.29 (0)	0.26 (0)			
Ser			1.18 (2)				1.95 (2)	1.42 (1)	1.20 (1)		0.85 (1)	0.27 (0)
Glx			1.07 (1)	0.89 (1)		0.86 (1)		1.62 (1)	0.39 (0)			
Pro			0.25 (0)	0.95 (1)			1.03 (1)	1.40 (1)	2.23 (2)	1.04 (1)		1.01 (1)
Gly			0.72 (0)	0.20 (0)			0.43 (0)	0.78 (0)	1.53 (1)			
Ala	1.0 (1)		0.76 (1)				1.40 (2)	0.27 (0)	0.23 (0)	1.01 (1)		0.94 (1)
Val		1.0 (1)		0.83 (1)			1.0 (1)	3.0 (3)				
Ile			0.53 (0)						1.0 (1)		1.0 (1)	
Leu			1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)		2.11 (2)	0.22 (0)			1.0 (1)
Phe										1.0 (1)		1.01 (1)
Lys			1.06 (1)			0.93 (1)		0.23 (0)				
Tyr				(1)		(1)	(1)	(1)	(1)	(1)	(1)	(1)
His	(1)	(1)	(1)		(1)				(1)			
sequence assignment	50–51	179–180	115–125	7–16	126–127	99–105	65–74	75–88	17–24	175–178	53–56	174–178
labeled residue	H51	H180	H121	Y12	H127	Y100	Y67	Y77	Y22 H24	Y176	Y54	Y176

[a] The peptides are numbered as in Figures 1 and 2. The theoretical compositions for the assigned peptide are given in parentheses. The compositions are given as ratios to the N-terminal hydrophobic amino acid. Peptide 8 is assumed to contain one residue of tryptophan.

Table II: Summary of pK and Reactivity Data for Con A Residues

residue	pK	r
Tyr (av)	9.04 ± 0.05	7.43 ± 0.25
His (av)	7.78 ± 0.07	1.33 ± 0.05
Lys (av)	8.99 ± 0.10	8.68 ± 0.59
Ala-1	8.19 ± 0.06	1.64 ± 0.06
Tyr-12	8.24 ± 0.17	5.12 ± 0.65
Tyr-22	9.32 ± 0.14	5.52 ± 0.65
His-24	8.45 ± 0.19	4.03 ± 0.47
His-51	7.16 ± 0.21	2.08 ± 0.19
Tyr-54	8.81 ± 0.20	6.25 ± 0.41
Tyr-67	9.07 ± 0.12	5.36 ± 0.57
Tyr-77	8.61 ± 0.14	9.07 ± 0.72
Tyr-100	8.08 ± 0.07	2.26 ± 0.10
His-121	7.47 ± 0.25	2.59 ± 0.28
His-127	8.02 ± 0.09	2.43 ± 0.13
Tyr-176	8.85 ± 0.08	16.9 ± 1.03
His-180	7.22 ± 0.20	1.64 ± 0.15

theoretical curves are reported in Table II. The reactivities, r, are expressed relative to that of the internal standard Gln-Gly, whose second-order velocity constant for reaction with Dnp-F is 0.838 M^{-1} min^{-1} (H. Kaplan, Y. K. Chan, and G. Oda, unpublished observations).

Discussion

By the chemical procedures described above, the pK values and reactivities of individual histidine and tyrosine residues can be determined, and the residues can be assigned within the protein sequence. Changes in the chemical properties of lysine residues have been successfully exploited to locate interaction sites involving lysine side chains on cytochrome c (Rieder & Bosshard, 1980). It is anticipated that similar effects may occur with interactions that involve histidine and tyrosine residues, and the technique should detect them.

The reagent used, Dnp-F, can also modify cysteine residues and the amino groups of lysine and N-terminal residues. We have previously determined average pK values for such residues in immunoglobulins with Dnp-F (Kaplan et al., 1980). However, proteins fully modified with Dnp-F are insufficiently soluble for proper proteolytic digestion. In this work, the amino groups were blocked with maleic anhydride after the trace labeling with [^{3}H]Dnp-F, prior to full modification by Dnp-F. The ϵ-[^{3}H]Dnp-lysine residues thus formed do not interfere with the determination of the histidine and tyrosine properties. For example, if a lysine that occurred in a tyrosine peptide were labeled, the final derivative would be a di-Dnp one, which would have significantly different mobility from the desired maleyl-lysine–Dnp-tyrosine peptide. In this study, advantage was taken of the formation of ϵ-[^{3}H]Dnp-lysine to measure the average properties of the lysine residues. In the case of cysteine residues, which Con A lacks, it should be straightforward to obtain pK and reactivity information for individual residues in the same manner as for tyrosine and histidine residues.

The data obtained for the residues of Con A fit titration curves reasonably well, though the data for the averaged histidine and tyrosine residues obtained by direct hydrolysis of the modified protein are superior to those for individual residues. The lack of obvious discontinuities in the curves is to some degree surprising, since there are several structural changes that Con A undergoes in the pH range employed. These are (a) association of dimers into tetramers between pH 5 and pH 7 (McKenzie et al., 1972) possibly associated with histidine residues H127 and H51 (Reeke et al., 1975), (b) a CD change in the tyrosine region (McCubbin et al., 1971), in the same pH range, which may be associated with the formation of tetramers or with ionization of a group near tyrosine residues, possibly H205 which neighbors Y12 and Y100, (c) formation of aggregates on being allowed to stand at room temperature above pH 7 (McKenzie et al., 1972), and (d) irreversible alkaline denaturation accompanied by CD changes between pH 8 and pH 9 (Pflumm & Beychok, 1974). The first two effects probably occur at too low a pH to be picked up by the chemical modification method. The third was minimized by adjustment to the final pH being made immediately prior to the [^{3}H]Dnp-F being added; hence, the reaction probably proceeded to a large degree before aggregation occurred. The fourth effect is the most likely one to be observed in the experiments; its relation to individual histidine or tyrosine residues is discussed below.

There has been one previous study of the chemical properties of the six histidine residues in Con A, the proton NMR work of Carver et al. (1977). The pH range used was 3–5; hence, pK data were not obtained. Three groups of histidine residues were apparent: (a) the metal binding residue H24, (b) residues H51, H127, and H180, and (c) residues H121 and H205. At higher pH values (J.-R. Brisson, G. E. D. Jackson, and N. M. Young, unpublished observations), the signals of the latter two groups merge and the average pK of the five is 7.57 ± 0.02. This value is in reasonable agreement with the average pK of

the six residues, 7.78, obtained here (Table II), taking into account the high pK found for the sixth histidine, H24.

In general, the histidine residues show less variation in properties, except for the metal binding residue H24, than the tyrosine residues. The reactivity range is just over 2-fold for the histidines but 7-fold for the tyrosines. The histidine reactivities are above the reactivity expected for a free imidazole group, as found previously in immunoglobulins (Kaplan et al., 1980), which points to significant interactions of these side chains with neighboring structural elements. The properties of the individual residues summarized in Table II can be considered in light of the structure determined for Con A by X-ray crystallography. Some of the residues play roles in the various binding sites on the protein, while others occur at monomer contact areas either within the basic dimer unit or at the dimer–dimer interface. Of the 13 histidines and tyrosines, only H24, H180, Y54, and Y67 are homologous to residues in other lectins. The present preliminary interpretation of the data is based on the structural analyses of Reeke et al. (1975) and a model constructed from their coordinates. The participation of the residues in hydrogen bonds and the exposure of the residues, particularly the hydroxyl groups of the tyrosine side chains, are chiefly considered.

The carbohydrate binding site of Con A includes two tyrosine residues, Y12 and Y100 (Hardman, 1979), and one histidine residue, H205, clustered together. Unfortunately, the properties of H205 could not be measured because peptides were not consistently obtained from its region. Its ionization may influence the neighboring tyrosine residues, possibly giving rise to the changes in CD spectra that occur in the pH range 5–7. Residues Y12 and particularly Y100 are the least reactive and lowest in pK of the seven tyrosine residues, although their hydroxyl groups appear to be well exposed on the surface of the protein. It is possible that charge-transfer effects between the two tyrosines are reducing both their pK's and reactivities. It should be noted that in this region contacts occur between tetramers in the crystal lattice (Reeke et al., 1975); hence, the X-ray-derived structure may differ slightly from the solution form, around these tyrosines.

In contrast to these relatively exposed tyrosines, Y54 lies within the hydrophobic pocket that is a major feature of the Con A molecule. Its hydroxyl forms a hydrogen bond to the peptide backbone at L81. The residue is of average pK and reactivity among the seven. This suggests that the reagent Dnp-F is not bound strongly by the hydrophobic pocket; i.e., the reagent does not behave as a weak affinity label for this region.

The final residue involved in a Con A binding site is H24, a ligand to transition metals in the double metal site (Hardman et al., 1982). The data obtained are of poorer quality for this residue, and its interpretation is also made more difficult by uncertainty as to the state being examined. For, although it is regarded as a Mn^{2+} binding site, this subsite is more often occupied by Mg^{2+} in native Con A (Young, 1983). The preparation of Con A used was not treated to alter the natural metal content; hence, at least two-thirds of the subsites would be occupied by Mg^{2+}. The interactions of the histidine side chains with the two metals would be different, and hence, their reactivities with Dnp-F would also be different. The pK and reactivity values must represent averages of H24 residues in Mg^{2+} and Mn^{2+} sites. The values found for H24 are higher than those of the other histidines, which is unexpected for histidines interacting with metals. It is possible that the neighboring acidic residues, D10 and D19, play a greater role than the metals in determining the properties of H24. Alternatively, the properties may be reflecting larger scale changes in the protein's structure. The pK value of 8.4 is in the pH range where irreversible denaturation is reported to occur (Pflumm & Beychok, 1974). The histidine may be acting as a reporter group for this change, going from a species of low reactivity, due to the interaction with the metal atom, to one of higher reactivity in the denatured state. This behavior would resemble that of the N-terminal histidine of glucagon (Cockle et al., 1982), where the imidazole's properties reflect those of the N-terminal amino group.

The histidine and tyrosine residues that take part in the oligomer formation include Y176, which is by far the most reactive of the seven tyrosine residues. Its side chain interacts noncovalently with a small group of residues across the monomer–monomer interface, R90, A177, P178, and Y176 (Reeke et al., 1975). Since Dnp-F reacts with the negatively charged tyrosinate form, it is possible that the nearby positively charged arginine residue influences the reactivity of Y176. It is interesting that the close pair of tyrosines, Y12 and Y100, have low reactivities while the pair of Y176 residues have high reactivities. The hydroxyl groups of the former may be closer together, the residues being in a more parallel arrangement than that of the Y176 residues.

Three histidine residues, H51, H121, and H127, occur at the monomer interaction regions. The latter may form a H bond to M129 while interactions of H51 with S117 and H121 with S108 are possible (Reeke et al., 1975). The reactivities of the three residues are similar, and there is a small spread in pK values. Their environments appear similar both from the chemical and from the structural viewpoints. The H127 residues lie within a central pocket in the tetramer into which heavy atom derivatives could diffuse in the crystal (Reeke et al., 1975). They are evidently accessible to the reagent Dnp-F in solution, judging from their reactivity.

The remaining residues lie on the exterior of the subunit. They include the N-terminal alanine residue A1, which arises by an unusual postsynthetic modification (Carrington et al., 1985). It is not well seen in the X-ray structure, being quite mobile, and its pK and reactivity (above that of the internal standard Gln-Gly) accord with an exposed nature. The sixth histidine, H180, interacts with a glutamic acid residue, E87, yet is similar to H51 in its properties. Of the remaining three tyrosines, only Y22 has its hydroxyl group fully exposed on the surface. The hydroxyl of Y67 forms a H bond with the peptide N of the glutamine residue Q43 and is more inaccessible than that of Y77. The ionization of the hydroxyls of the latter two residues, particularly Y67, may also contribute to the alkaline denaturation phenomenon mentioned above. The properties of the three tyrosines are not very different, the most exposed residue, Y22, being almost identical with the least exposed, Y67.

This preliminary analysis explains several features of the data, but an explanation of the overall pattern of tyrosine reactivity is not apparent. Reaction of Dnp-F with histidine side chains involves the uncharged form of the imidazole ring, and H-bond interactions with neighboring residues obviously could influence its reactivity. Histidines in proteins in general have reactivities greater than that of a free imidazole, as seen with immunoglobulins (Kaplan et al., 1980). With tyrosine, it is the charged tyrosinate form that reacts with Dnp-F. H bonding to nearby residues will influence the properties of the hydroxyl form but not necessarily more than interactions with water molecules. Factors affecting the tyrosinate form would obviously include nearby charged groups such as in the case of the Y176–R90 relationship. Hence, quite different factors

would be important for the two types of residue. It is hoped that higher resolution data for the Con A structure analyzed by molecular graphics methods may resolve these factors better.

In conclusion, the experimental approach described allows description of the properties of individual tyrosine and histidine residues, and potentially cysteine residues, in proteins. This should make possible studies of the roles of these residues in interaction sites and contribute to understanding how protein structure can affect the properties of individual residues.

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Reassociation of Dimeric Cytoplasmic Malate Dehydrogenase Is Determined by Slow and Very Slow Folding Reactions†

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ABSTRACT: Malate dehydrogenase occurs in virtually all eucaryotic cells in mitochondrial and cytoplasmic forms, both of which are composed of two identical subunits. The reactivation of the mitochondrial isoenzyme has been the subject of previous studies [Jaenicke, R., Rudolph, R., & Heider, I. (1979) *Biochemistry 18*, 1217–1223]. In the present study, the reconstitution of cytoplasmic malate dehydrogenase from porcine heart after denaturation by guanidine hydrochloride has been determined. The enzyme is denatured by >1.2 M guanidine hydrochloride; upon reconstitution, ~60% of the initial native enzyme can be recovered. The kinetics of reconstitution after maximum unfolding by 6 M guanidine hydrochloride were analyzed by fluorescence, far-ultraviolet circular dichroism, chemical cross-linking with glutaraldehyde, and activity measurements. After fast folding into structured intermediates (<1 min), formation of native enzyme is governed by two parallel slow and very slow first-order folding reactions ($k_1 = 1.3 \times 10^{-3}$ s^{-1} and $k_2 = 7 \times 10^{-5}$ s^{-1} at 20 °C). The rate constant of the association step following the slow folding reaction (determined by k_1) must be $>10^6$ M^{-1} s^{-1}. The energy of activation of the slow folding step is of the order of 9 ± 1 kcal/mol; the apparent rate constant of the parallel very slow folding reaction is virtually temperature independent. The intermediates of reassociation must be enzymatically inactive, since reactivation strictly parallels the formation of native dimers. Upon acid dissociation (pH 2.3), ~35% of the native helicity is preserved, as determined by circular dichroism. Despite the residual structure, reconstitution after short-term acid dissociation (5 min) is governed by the same slow and very slow folding reactions as those observed after unfolding by guanidine hydrochloride. After long-term acid incubation (24 h), both the rate and yield of reactivation decrease, presumably due to a conformational rearrangement within the acid-dissociated monomers.

The mechanism of folding of small monomeric proteins such as bovine pancreatic trypsin inhibitor or ribonuclease has been investigated in considerable detail [cf. Kim & Baldwin (1982)]. In the case of larger multidomain or oligomeric proteins, the acquisition of the native three-dimensional structure is more complex since chain folding, domain pairing, and subunit association need to be properly coordinated (Jaenicke, 1984). As a consequence, the yield of reconstitution is generally below

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