VARIABLE-TEMPERATURE FOURIER TRANSFORM INFRARED INVESTIGATION OF THE SECONDARY STRUCTURE OF CONCANAVALIN A

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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March, 1995

ABSTRACT

Changes in the secondary structure and aggregation of concanavalin A (Con A) were investigated by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. The secondary structure of Con A has been shown to be affected by factors such as temperature, pH, ionic strength, pressure and the presence of sugars. DSC studies indicated that the thermal stability of Con A is enhanced in the presence of salts, sugars and by pH. Detailed examination of the conformation-sensitive amide I band in the FTIR spectrum of Con A revealed that concentration, sugars, metal ions and pressure (range from 0 -14.4 kbar) had no effect on the structural conformation of Con A at room temperature. Variable-temperature FTIR studies of the thermal stability of Con A as a function of pH showed that maximum thermal stability is achieved under alkaline conditions (pH 8 - 10). It was also found that sugars enhanced the thermal stability of Con A to varying degrees and that the stabilizing effect of the sugar was not dependent on the sugar binding specificity of the lectin.

RESUME

Les changements dans la structure et l'agrégation de la concanavaline A (Con A) ont été étudiés par calorimétrie différentielle (DSC) et par spectroscopie infrarouge en transformée de Fourier (FTIR). La structure secondaire de la Con A est affectée par différents facteurs tels que la température, le pH, la force ionique, la pression et la présence de sucres. Les études DSC montrent que la stabilité de la Con A est accrue par la présence de sels, de sucres et le pH. Un examen plus approfondi de la bande amide I dans le spectre FTIR de la Con A révèle que la concentration en sucres et en ions métalliques ainsi que la pression (variant de 0 à 14.4 kbar) n'ont aucun effet sur la conformation de la Con A à la température ambiante. Des études portant sur la stabilité de la Con A selon la température en fonction du pH et des sucres présents, réalisées par FTIR à différentes températures, montrent que la stabilité maximum est obtenue dans des conditions alcalines (8 - 10). De plus, les sucres augmentent la stabilité thermique de la Con A à différents degrés et cet effet de stabilisation est indépendante de la fixation spécifique du sucre sur les lectines.

ACKNOWLEDGMENTS

I am sincerely grateful to my supervisor, Dr. Ashraf A. Ismail for his guidance, precious advise and support throughout this study.

I would like to extend my sincere gratitude to Ms. Jacqueline Sedman and Mr. Gary Emo for their help throughout my study. Thanks to all the graduate students and staff of the department for their friendship throughout my student life at Macdonald campus, especially thanks to Véronnique for her kind help on translating the abstract. Without their friendship this thesis would have never been possible.

I would like to express my sincere appreciation to my family for their support throughout my studies. My deep gratitude is extended to my parents who always encourage me to pursue the road of knowledge and to my brother and sister-in-law, who gave me the strength to complete this work.

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LIST OF ABBREVIATIONS

Arb	--	Arabinose
Con A	--	Concanavalin A
def	--	Deformation
DSC	--	Differential Scanning Calorimetry
Fru	--	Fructose
FTIR	--	Fourier Transform Infrared
Gla	--	Galactose
Glu	--	Glucose
Lac	--	Lactose
Mal	--	Maltose
Man	--	Mannose
Str	--	Stretching
Sur	--	Sucrose
VT-IR	--	Variable-Temperature Infrared

CHAPTER 1. INTRODUCTION

Proteins are very important ingredients in food products. The specific responses of food proteins to applied forces during processing and storage are directly related to their structure and conformation at the molecular level. One of the characteristics of proteins is that their native conformation can be converted to the denatured state by changing the chemical or physical conditions of the medium. Thus the determination of protein secondary structure in solution remains an area of ongoing biophysical importance.

Lectins are an important group of proteins having antinutritional properties and can bind to carbohydrates, agglutinate cells or precipitate polysaccharides and glycoproteins. Concanavalin A (Con A), which is from jack bean, is the most well-known plant lectin (Brown & Hunt, 1978). It binds specifically to carbohydrate-containing groups on the surface of a variety of eukaryotic cells, resulting in cell agglutination. Con A has found a variety of uses in biochemical and immunochemical studies. It requires the presence of two metal ions, usually Mn^{2+} and Ca^{2+}, for optimal saccharide binding (Yariv et al. 1968).

Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) are two suitable techniques for the study of protein conformation, and especially for the study of thermal and structural transitions of food proteins during preparation, processing and storage.

Infrared spectrometry is a particularly suitable technique to study protein conformation and protein-ligand interaction (Susi, 1969; Amey & Chapman, 1983). Many studies, both experimental and theoretical, have shown that the amide I band, usually observed between 1600 and 1700 cm^{-1}, is the most important region for the study of secondary structure of proteins. The position of the amide I band absorption may be related to the α-helical, β-strand, and "random" coil conformations in the protein (Krimm, 1962; Susi et al. 1967; Koenig et al. 1980). The amide I mode is composed of 80% C=O stretching arising from the peptide bond; this vibrational mode is very sensitive to changes in the nature of the hydrogen bond and therefore leads to characteristic infrared bands from the different conformations of the peptide backbone (Krimm, 1962; Susi et al. 1967; Timasheff et al., 1967; Koenig & Tabb, 1980).

Differential scanning calorimetry (DSC) has emerged as the technique of choice for the study of the thermal transitions of food proteins. The use of thermal analysis, and in particular, differential scanning calorimetry (DSC), to monitor changes in the heat capacity of a protein system during heating provides a relatively simple and convenient technique for following protein denaturation (Rüegg et al., 1977; Donovan et al., 1975). In this technique, a sample and an inert reference are maintained at the same temperature while the temperature of both is gradually raised, usually at a programmed linear rate. Any thermally induced changes occurring in the sample are then recorded as differential heat flow displayed normally as a peak in a thermogram with respect to time or temperature, yielding a value for the enthalpy associated with the heat induced transitions of state of the

sample (Scheidler & Steim, 1975; Ashe & Steim, 1977; Jackson & Sturtevant, 1977; Jackson et al., 1973).

Con A was the first lectin whose native structure was determined by X-ray crystallography, and the structure has been refined to 1.75 Å, It consists of a tetramer (Hardman et al., 1982). The basic subunit has a molecular weight of 25,500 daltons and associates to form predomiantly dimers in solution at pH 5 and tetramers above pH7. The most characteristic feature of the secondary structure of Con A as seen from the X-ray data is that the tetramer has about 60% overall β-structure. The protein also contains about 40% of turns and loops. To have binding activity, each monomer needs one transition metal ion, Mn^{2+} in the native form, and one calcium ion to give the necessary conformation of the polypeptide chain at the binding site which is formed from four peptide loops. Earlier infrared spectroscopic studies of the structure of Con A have been published and the results are consistent with the predominance of antiparallel β-sheet structure (Painter, et al., 1975; Ockman, 1981; Purcell et al., 1984; Byler et al., 1986).

In this work, FTIR spectroscopy and DSC are employed to probe the changes in the secondary structure of Con A as a function of varying physico-chemical conditions. Furthermore, the effect of sugar on the thermal stability of Con A will be examined in some detail.

CHAPTER 2. LITERATURE REVIEW

2.1 PROTEIN ARCHITECTURE

There are several levels of protein structure and different types of interactions that maintain such structures. The primary structure is the amino acid residue sequence in the peptide chain. The secondary structure is the intramolecular rearrangement wherein domains of high symmetry are produced in sections of the peptide chain. The tertiary structure is the intramolecular arrangement of the above domains with respect to each other. Quaternary structures are formed by the aggregation of individual peptide chains (subunits) to form ordered supramolecular structures (Franks, 1988). FTIR spectroscopy is well suited for the study of secondary structure of proteins.

Compared to the covalent bond energies involved in the primary structure, the secondary structure relies on weak, hydrogen-bonding interactions. It originates from the stereochemistry of the peptide bond (shown in Fig.1). The six atoms within the shaded area lie in one plane because the C-N bond has some double bond character. Rotation can occur about the C-C and C-N bonds, and successive residues in the polypeptide chain are characterized by a pair of torsional angles.

2.1.1 The α-helix

Apart from possible interactions between amino acid side chains, the

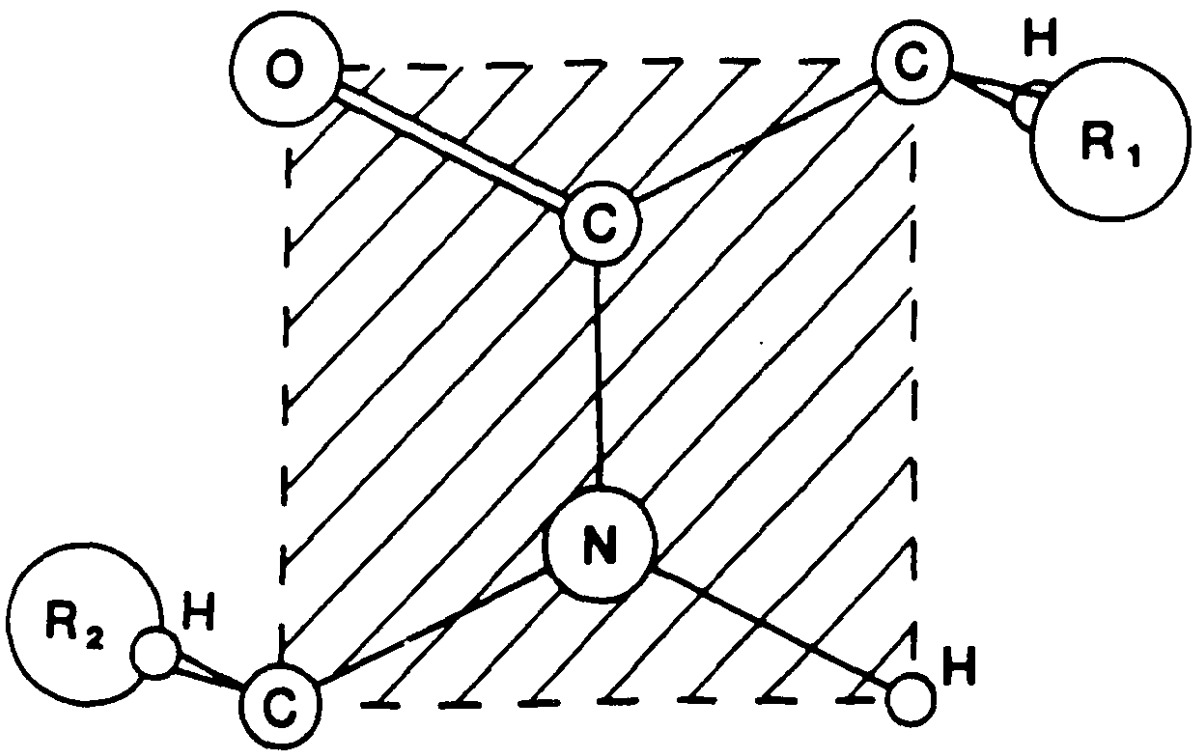

Figure 1. Stereochemistry of the *Trans*-Peptide Bond, as Found in Proteins (from Franks, 1988)

α-helix is the form of lowest free energy. It therefore forms spontaneously, especially in fibrous proteins, which usually contain repeating patterns in their primary sequences (Fig. 2).

2.1.2 The β-pleated sheet

Another high-symmetry form is the so-called β-structure, which is adopted by peptide chains that cannot easily form an α-helix. It is an extended, corrugated structure. The chains are linked laterally by hydrogen bonds to form sheets. Two possible configurations exist, depending on whether neighboring chains run parallel or antiparallel (Fig 3).

2.1.3 Turns

Tight turns in protein have long been recognized as important elements of secondary structure. The definition of turns has, however, been considerably modified and expanded to include several variations of the main scheme (Fig. 4) (Venlatacholam, 1968). In its most general sense, a turn is said to be generated whenever a polypeptide chain changes direction. A β-turn, more specifically, consists of a four amino acid residue segment with or without an internal hydrogen bond (Richardson, 1981; Rose et al., 1985).

2.2 LECTINS

Lectins have been defined by and considered mostly for their binding

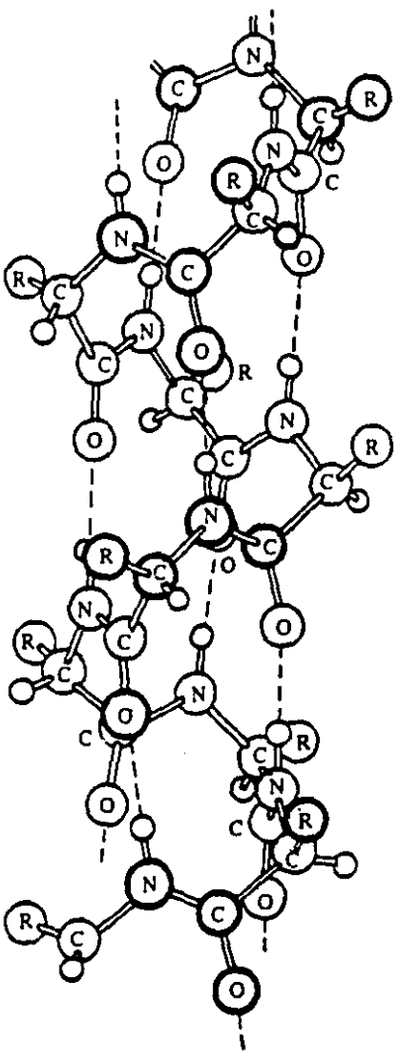

Figure 2. α-Helix Structure of Fibrous Proteins (from Franks, 1988)

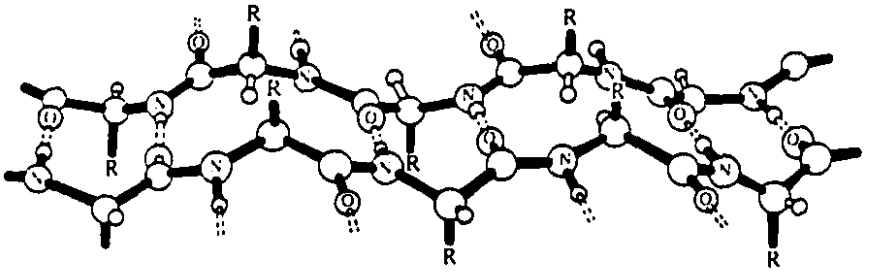

Figure 3. β-Sheet Structure (from Franks, 1988)

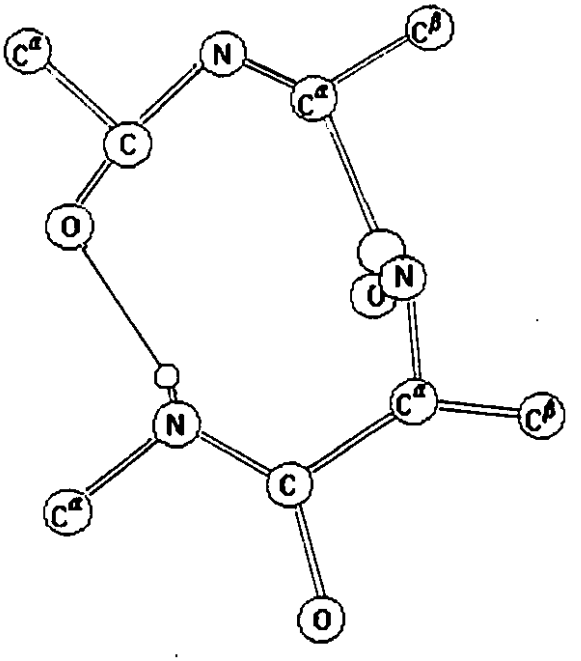

Figure 4. Diagram of a Typical Hydrogen-bonded Turn (from Rose et al., 1985)

to carbohydrate moieties. They are particularly useful for the separation of glycoproteins and glycopeptides and to probe changes in cell surface sugars during cell growth, differentiation, and malignancy. They have also been used to study the mitogenic stimulation of lymphocytes, which occurs at least partly through their binding to specific carbohydrate determinants present at the cell surface (Hume & Weidemann, 1980; Lis & Sharon, 1984; Sharon & Goldstein, 1986; Lis & Sharon, 1986; Franz, 1988; Josiane et al., 1990). They are being widely used for preparative and analytical purposes in biochemistry, cell biology, immunology, and related areas, particularly with glycoconjugates (Table 1). The list of applications of lectins is growing rapidly and the potential of these proteins is far from completely explored (Lis & Sharon, 1989).

The term "lectin" was first introduced by Boyd and Shapleigh (1954) to describe a class of proteins of plant origin which agglutinate cells and exhibit antibody-like sugar binding specificity. Now the definition adopted by the Nomenclature Committee of the International Union of Biochemistry defines a lectin simply as a carbohydrate-binding protein of nonimmune origin that agglutinates cells or precipitates polysaccharides or glycoconjugates. This definition implies that lectins are multivalent, that is, they possess at least two sugar binding sites which enable them to agglutinate animal and plant cells and/or to precipitate polysaccharides, glycoproteins, peptidoglycans, teichoic acids, glycolipids, etc. The sugar specificity of lectins is usually defined in terms of the monosaccharide(s) that inhibits lectin-induced agglutination or precipitation reactions (Lis & Sharon, 1989; Chowdhury & Weiss, 1975; Goldstein, 1979).

Table 1. Major Uses of Lectins

1. Isolation, purification, and structural studies of carbohydrate-containing polymers.
2. Investigation of complex carbohydrate structures on surfaces of animal cells, bacteria, and viruses and of subcellular particles.
3. Investigation of the architecture of cell surfaces and its change upon malignant transformation.
4, Blood typing, structural studies of blood group substances; identification of new blood types; diagnosis of secretors.
5. Isolation of lymphocyte subpopulations and a stem cell-enriched fraction of bone marrow suitable for transplantation.
6. Studies of the genetics, biosynthesis, and function of cell-surface glycoconjugates.
7. Mitogenic stimulation of lymphocytes; studies of events occurring upon initiation of cell division; studies on lymphokines; studies of chromosomal constitution of cells and detection of chromosomal abnotmalities.
8. Studies of specific carbohydrate binding sites on protein.

From Lis & Sharon, 1989

2.3 CONCANAVALIN A

Concanavalin A (Con A) was first isolated and crystallized by Sumner and Howell (1936), who established it to be the phytohemagglutin of the jack bean (*Canavalia ensiformis*), Con A exhibits a series of remarkable biological properties, making it the most intensively investigated plant lectin.

2.3.1 Structural Features of Con A

Con A consists of four carbohydrate-free subunits, Mr=26,500. As usually prepared, it contains a mixture of two forms, one consisting of intact protomers and another consisting of monomers containing two fragments of molecular weight 13,000 and 11,000 (Wang *et al.*, 1971). No amino acids are missing in a protomer consisting of these two fragments. Fragmentation is probably due to proteolysis between residues 118 and 111 (Fig. 5). Edmundson *et al.* (1971) found three chain fragments and from their results they concluded that the primary gene product was chain II (molecular weight 27,000) but that chains I (N-terminal, molecular weight 12,500) and III (C-terminal, molecular weight 14,000) originated from chemical or enzymatic hydrolysis of the intact chain. These results correspond roughly to those of Wang *et al.*(1971).

The three-dimensional structure of the Con A molecule was studied by X-ray crystallography of single crystals (Greer et al., 1970) at low resolution (4-4.25 Å) by Quiocho et al. (1971) and Hardman et al. (1971) and then at higher resolution (2-2.4 Å) by Edelman et al. (1972) and

1 10
ALA-ASP-THR-ILE-VAL-ALA-VAL-GUL-LEU-ASP-THR-TYR-PRO-ASN-THR-ASP-ILE-GLY-

20 30
ASP-PRO-SER-TYR-PRO-HIS-ILE-GLY-ILE-ASP-ILE-LYS-SER-VAL-ARG-SER-LYS–LYS-THR-

40 50
ALA-LYS-TRP-ASN-MET-GLN-ASP-GLY-LYS-VAL-GLY-THR-ALA-HIS-ILE-ILE-TYR-ASN-

60 70
SER-VAL-ASP-LYS-ARG-LEU-SER-ALA-VAL-VAL-SER-TYR-PRO-ASN-ALA-ASP-ALA-THR-

80 90
SER-VAL-SER-TYR-ASX-VAL-ASP-LEU-ASX-ASX-VAL-LEU-PRO-GLN-TRP-VAL-ARG-VAL-

100
GLY-LEU-SER-ALA-SER-THR-GLY-LEU-TYR-LYS-GLU-THR-ASN-THR-ILE-LEU-SER-PHE-

110 120
SER-TRP-THR-SER-LYS-LEU-LYS-SER-ASX-SER-THR-HIS-GLX-THR-ASX-ALA-LEU-HIS-PHE-

130 140
MET-PHE-ASN-GLN-PHE-SER-LYS-ASP-GLN-LYS-ASP-LEU-ILE-LEU-GLN-GLY-ASP-ALA-

150 160
THR-THR-GLY-THR-ASP-GLY-ASN-LEU-GLU-LEU-THR-ARG-VAL-SER-SER-ASN-GLY-SER-

170 180
PRO-GLU-GLY-SER-SER-VAL-GLY-ARG-ALA-LEU-PHE-TYR-ALA-PRO-VAL-HIS-ILE-TRP-

190 200
GLU-SER-SER-ALA-(THR.ALA.SER.VAL)-PHE-GLU-ALA-THR-PHE-(THR.LEU.VAL)-ILE-LYS-

210
SER-PRO-ASP-SER-HIS-PRO-ALA-ASP-GLY-ILE-ALA-PHE-PHE-ILE-SER-ASN-ILE-ASX-SER-

220 230
SER-ILE-PRO-SER-GLY-SER-THR-GLY-ARG-LEU-LEU-GLY-LEU-PHE-PRO-ASP-ALA-ASN

Figure 5. Tentative Amino Acid Sequence of Concanavalin A (from Reeke et al., 1974)

Hardman and Ainsworth (1972a). The arrangement of the four protomer subunits in the tetramer is shown schematically in Figs. 6 and 7.

The special relationships of the transition metal and calcium ions with the protein are represented in Figs. 7 and 8. The *myo*-inositol binding subsite has been studied by Hardman and Ainsworth (1972b); reaction with *myo*-inositol produces only a small conformational change of the Con A molecule. An important feature of the secondary structure is that on binding with a specific sugar (α-methyl-D-mannose) a conformational change takes place which may be the first step in the chain reactions of haemagglutination and mitogenesis. It is also interesting that the transition metal and Ca^{2+} ions are situated close together at a distance of about 20 Å from the saccharide binding site (Figs. 7 and 8)

2.3.2 Carbohydrate-Binding Properties of Con A

The carbohydrate-binding properties of Con A were first recognized by Sumner and Howell (1936) Early studies by Goldstein and colleagues (1965a,b) indicated that con A would bind α-glucosyl and α-N-acetylglucosaminyl groups and would precipitate branched polysaccharides containing these sugar units. The carbohydrate-binding specificity of con A has been discussed in detail by Goldstein *et al.* (1974). A summary of the results of such studies is presented in Table 2.

Mannose, in its α-anomeric form, is the monosaccharide most complementary to the con A sugar binding site and, although there appear

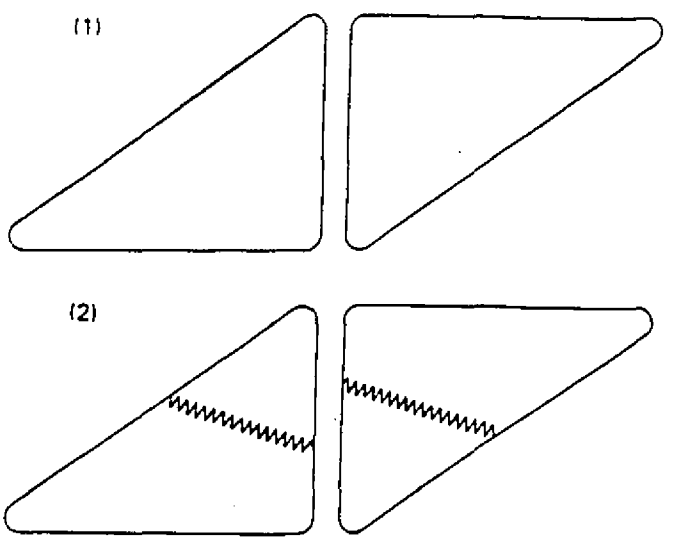

Figure 6. Subunit Structure of Concanavalin A
(from Ainsworth, 1972)

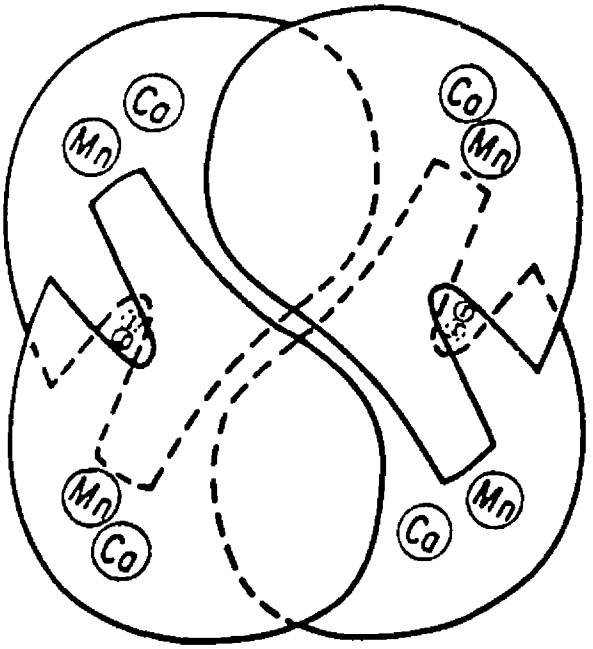

Figure 7. Schematic Representation of the Tetrameric Structure of Concanavalin A (from Reeke et al., 1973)

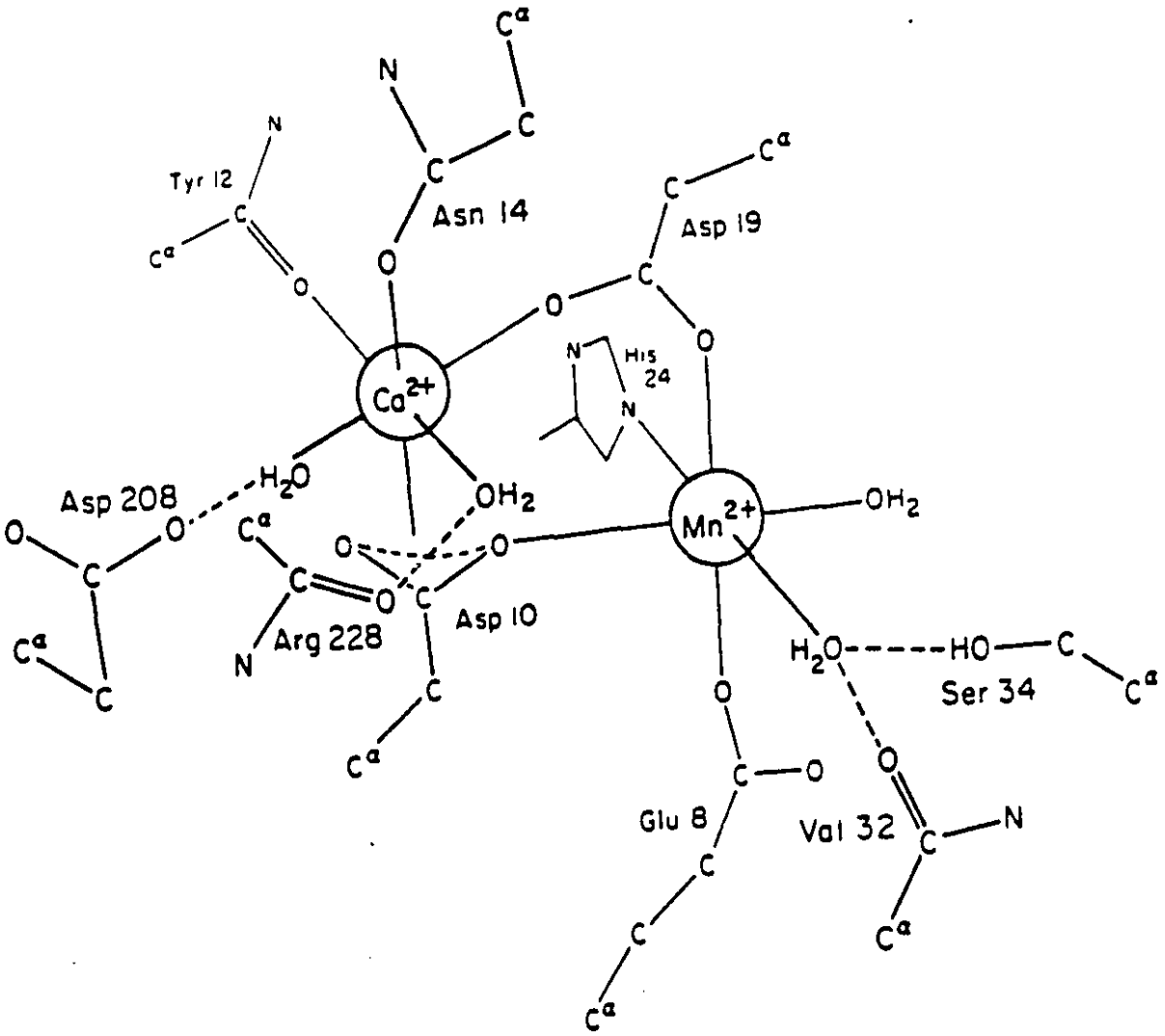

Figure 8. Schematic Drawing of the Metal Coordination Sites in Concanavalin A (from Reeke et al., 1974)

TABLE 2. Inhibition of Concanavalin A-Dextran 1355-S Precipitation by Mono- and Oligosaccharide and Some of Their Derivatives

Inhibitor	Relative inhibitory potency[a]
Glucose	0.22
Mannose	1.0
Galactose	0.5
Maltose	<0.04
Sucrose	0.19
Methyl α-mannopyranoside	7.33
Methyl α-glucopyranoside	1.76
Methyl *N*-acetyl-α-glucosaminide	0.88
Methyl α-fructopyranoside	<0.03
Methyl β-fructopyranoside	5.17

a Data taken from Goldstein et al. (1965, 1967), So and Goldstein (1967, 1969), Smith and Goldstein (1967), and Poretz and Goldstein (1970). Mannose is normalized to 1.0.

to be loci in the protein-combining site capable of interacting with each hydroxyl group of this sugar, the hydroxyl groups most critical for binding to the lectin are those at positions C-3, C-4, and C-6 of the pyranosyl ring system (see Fig. 9) Any modification of these hydroxyl groups, for example, a change in configuration at C-4 to give talose or conversion to a deoxy sugar (e.g., 6-deoxymannose) or to an *O*-methyl sugar (e.g., 6-*O*-methylmannose), drastically reduces or abolishes interaction with the protein. Con A will tolerate considerable variation at the C-2 position of the D-pyranose ring. Thus 2-deoxy and 2-O-methylglucose and mannose all bind to Con A; N-acetylglucosamine also binds but with one-half the affinity of glucose. The *manno*-configuration, with an axial hydroxyl group at C-2, binds to con A with higher affinity than the *gluco*-configuration with an equatorial hydroxyl group in this position. Hapten-inhibition studies with a series of deoxy, fluoro, and *O*-methyl derivatives of glucose and mannose have permitted a more penetrating analysis of the binding mechanism, e.g., a determination of whether it is the *hydrogen* or *oxygen* atom of each hydroxyl group of these sugars that may be involved in H-bonding to Con A (Goldstein et al., 1974; Poretz and Goldstein, 1970; So and Goldstein, 1967). Fig. 9 summarizes these results. The contribution of each hydroxyl group of methyl a-mannoside to the stabilization of the sugar-con A complex has been estimated (Poretz and Goldstein, 1970) and confirmed by the calorimetric measurements of Munske and his colleagues (1984). Certain sugars, e.g., aldopentose, arabinose, and the ketohexose fructose, in their five-membered furanoid ring form, also bind to Con A (So and Goldstein, 1969b). This is because the dispositions of the C-2, C-3, and C-5 hydroxyl groups of the arabinofuranosyl group and the C-3, C-4, and C-6 hydroxyl

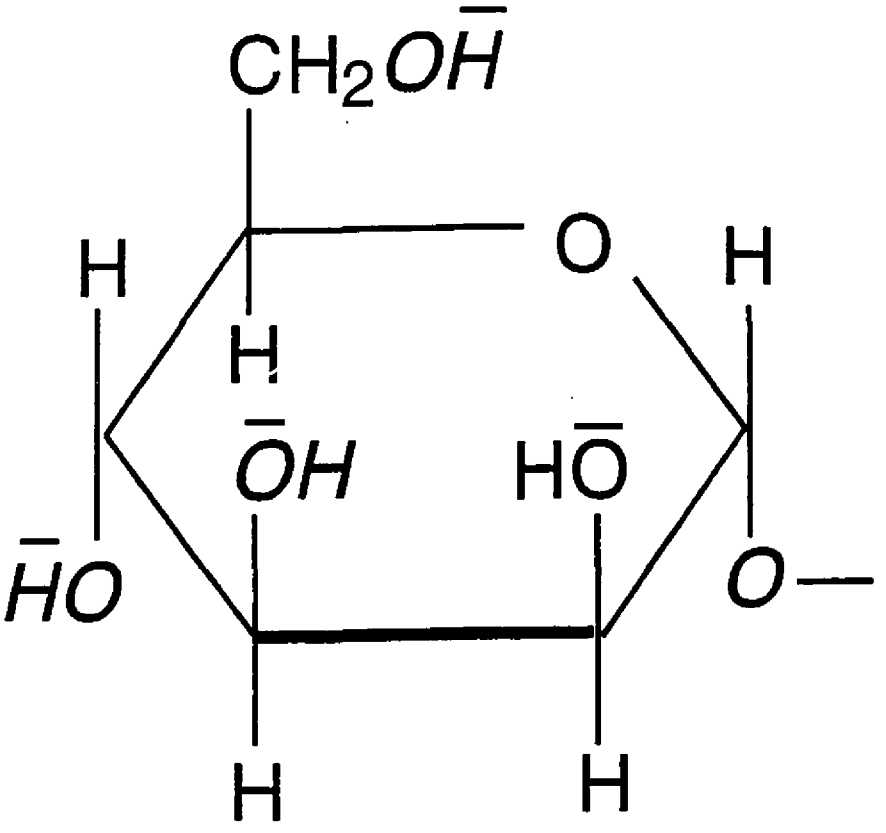

Figure 9. Carbohydrate-binding Specificity of Concanavalin A. The hydroxyl groups of the α-mannopyranosyl group that are most critically involved in binding to Con A are italicized. Hydrogen and oxygen atoms believed to participate in hydrogen bonding are overscored. (from Poretz and Goldstein, 1970)

units of the fructofuranosyl unit are similar to the orientation of the hydroxyl groups at C-3, C-4, and C-6 of the mannopyranosyl residue (Fig. 10).

Among a large number of oligosaccharides tested for their capacity to inhibit con A-polysaccharide precipitation, only those containing nonreducing terminal, α-linked glucose, mannose, or N-acetylglucosamine groups were active. Thus maltose, isomaltose, Mana1,6Glc, and glcNAc-a1,6GlcNAc acted as inhibitors (Goldstein *et al.*, 1965b; Smith and Goldstein, 1967). A notable exception was the disaccharide sophorose (Glcb-1,2Glc), which was shown to bind to Con A via the three critical hydroxyl groups at C-3, C-4, and C-6 of the *reducing* glucose unit (Goldstein *et al.*, 1967). This observation led to the important finding that α-mannosyl residues substituted at the C-2 position as occur in glycoproteins also may serve as receptor sites for con A (Goldstein *et al.*, 1973), and *N*-asparagine-linked glycoproteins, also binds to Con A with high affinity (Kaifu *et al.*, 1975).

2.3.3 Kinetics of Concanavalin A-Sugar Interaction

Con A binds low molecular weight carbohydrate ligands via a monophasic process characterized by second-order rate constants (at 25°C) of 10^4 - 10^5 M^{-1} sec^{-1} (Farina and Wilkins, 1980), which are several orders of magnitude below what one would expect for a diffusion-controlled process. This result has been taken to mean that severe steric constraints impede association, or that an unstable complex that initially forms at a

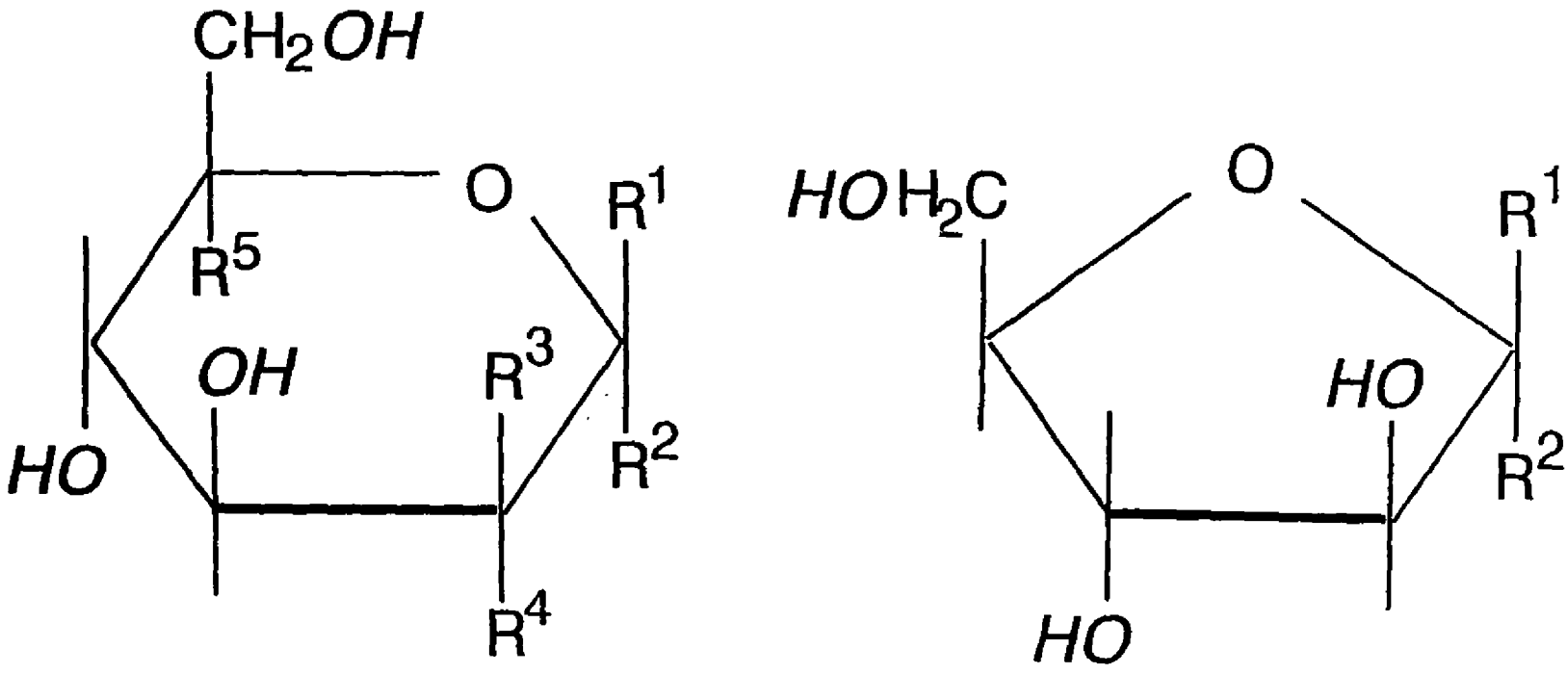

Figure 10. Configurational Relationships among Furanoid and Pyranoid Sugars that Interact with Concanavalin A. Hydroxyl groups critical for binding to Con A are italicized. Note the relationship between the hydroxyl groups in the furanoid and pyranoid systems. (from Academic Press)

diffusion-controlled rate is subsequently stabilized by a slow, rate-determining conformational change. Mannose oligosaccharides containing α-(1-2)-glycosidic bonds bind to Con A via multiphasic processes when the oligosaccharide contains more than one glycosyl residue that can interact with the primary carbohydrate binding site (Van Landschoot et al., 1980). Usually, the number of phases that can be discerned when Con A reacts with an oligosaccharide ligand is equivalent to the number of glycosyl residues in the ligand that are recognized by the primary glycosyl binding site. The multiphasic time course for binding of oligosaccharide to Con A may be rationalized in terms of a model first proposed by Williams et al. (1981) where the unsymmetrical oligosaccharides can bind to Con A in more than one way. In the initial rapid phase (second-order rate constant of 10^4-10^5 M^{-1} sec^{-1}) all possible complexes form in amounts determined by the relative rate constants of association. In the subsequent slow phases, the complexes equilibrate according to the relative values of the equilibrium constant for formation of each complex. Further studies (William et al., 1981) of the binding of disaccharides to Con A have demonstrated that the lectin is capable of extended interactions with groups on the second glycosyl residue of certain disaccharides. An axial orientation at the C-2 hydroxyl group of the second glucopuranosyl residue in α-1-2-linked *p*-nitrophenyl glycosides appears to be the most important determinant of the extended interaction (Williams et al., 1981).

2.3.4 Nonpolar Binding Sites of Concanavalin A

Three classes of nonpolar binding sites have been identified on the

Con A molecule; a site adjacent to the carbohydrate-specific site that interacts specifically with the phenyl groups of phenyl β-glucosides and mannosides (Poretz and Goldstein, 1971); a single high-affinity binding site in native and succinyl Con A that interacts with a fluorescent hydrophobic probe (Roberts and Goldstein, 1983a) and four low-affinity sites (one per subunit) that interact with ligands such as tryptophan and indoleacetic acid (Edelman and Wang, 1978); and a nonpolar binding cavity that appears to be present only in the crystalline state and binds relatively nonpolar molecules such as *o*-iodobenzoic acid, *p*-hydroxybenzoate, phenyl-phosphate, and dimethyl-mercury.

CHAPTER 3. THE STUDY OF THERMAL STABILITY OF CONCANAVALIN A BY DIFFERENTIAL SCANNING CALORIMETRY

3.1 OBJECTIVES

The objective of this study is to monitor the structural and thermal changes induced in proteins at various concentrations, ionic strengths and pH as a function of temperature and time by differential scanning calorimetry.

3.2 DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Differential scanning calorimetry is a particularly pertinent technique for studying the behaviour of proteins in foods because of its inherent utillisation of heat, which probably constitutes the commonest form of processing treatment applied to food systems. Thus, basic information, such as temperature and rates of the denaturation undergone by proteins during cooking, can be obtained under comparable conditions. In addition, DSC can be employed to monitor indirectly the effects of other processing operations, such as freezing, drying and mixing, by following the resultant changes in the thermal behaviour of the protein.

The parameters that are most often used to describe the thermal behaviour of proteins as ascertained by DSC analysis include the peak temperature of denaturation (Td), the start of denaturation (Ts), the enthalpy

change (ΔH) and the width at half-peak height (Tw). The latter value gives an indication of the cooperativity of the transition: if denaturation occurs within a narrow range (small width), the transition is considered highly cooperative.

The thermally induced process exhibited by most proteins and detectable by DSC is the structural melting or unfolding of the molecule, analogous in many ways to the phase transitions undergone by macroscopic systems. In proteins this is usually equated with the denaturation of the molecule, defined as the transformation from the native or ordered to the denatured or disordered state.

From earlier DSC studies of proteins, it was found that at low water content, where only hydration water is present, the temperature of denaturation is very dependent upon concentration (Wright, 1982). In samples containing less than 30% water, the water is distributed non-uniformly throughout the sample or, at least, is localized upon heat treatment and therefore has restricted access to the entire protein. This has a marked effect on the profile of thermal denaturation (Hägerdal and Martens, 1976). DSC studies on the protein constituents in egg white, lysozyme and ovalbumin, were reported by Hegg et al. in 1979. The denaturation of ovalbumin has been studied by DSC with the objective of clarifying the relationship between its heat-induced precipitation and gelation. The protein displayed optimum thermal stability in the pH range 6 - 10 with a denaturation temperature of approximately 79 ^{o}C. This decreased gradually to 62 ^{o}C at pH 3 and 73 ^{o}C at pH 11.

It is well known that sugars may protect proteins against loss of solubility during drying and may also inhibit heat coagulation. DSC studies revealed that denaturation of ovalbumin in urea solutions was reduced in the presence of sucrose (Donovan et al., 1975; Back et al., 1979). At the level of 10% addition of sucrose, the Td of the protein was shifted to higher temperatures by about 2 ^{o}C. Greater stabilization was apparent as sugar levels were increased, with the result that at a concentration of 50% sugar, the denaturation temperature had increased by between 9 and 17 ^{o}C depending upon the specific sugar and protein in question. Table 3 lists some sugars that have been shown by DSC to thermally stabilize proteins (Back et al., 1979).

In addition to sugars, salts have also been found to impart stabilizing effects on proteins. For example, the thermal stability of vicilin increased when mixed with salts in increasing molal concentration (Back et al., 1979). DSC studies have shown that addition of NaCl, the most commonly used food grade salt, results in increased stability of both vicilin and legumin, although the relative increase in Td values is not the same for the two (Lim, 1976: Von Hippel & Schleich, 1969). There does not appear to be any change in the peak areas associated with these transitions; ΔH values, however, were not calculated due to the overlapping of the two endotherms in some salt environments. For vicilin isolated from faba bean, a similar increase in Td has been reported with no significant change in ΔH in the range between 0.2 and 1.0 M NaCl. With isolated legumin, however, both Td and denaturation enthalpy increased with increasing NaCl concentration;

TABLE 3. Increase in Tm[a] of Some Proteins in the Presence of Various Sugars As Measured by DSC

Protein (pH7)	Sugar (50%)	Increase in Tm (^{O}C)
Lysozyme	Sucrose	14.5
	Glucose	15
Conalbumin	Sucrose	9
	Glucose	11
Ovalbumin	Sucrose	11
	Glucose	11.5
	Fructose	12
	Mannose	12.5
	Maltose	10
	Dextran	6.5

Data from Back et al., 1979

a: Tm for the maximum temperature of protein under stable state.

it was felt that the ΔH values are simply a function of the Td values. This improved stability associated with higher NaCl concentrations has also been noted for other plant protein isolates including soybean and safflower (Arntfiekd et al., 1989). The effect that denaturation has on the ability of a protein to bind water has also been investigated using DSC. Berlin et al. reported that, in the case of whey proteins, there was no significant difference in the water binding of the proteins before and after thermal denaturation. Luescher et al. observed the release of primary hydration water upon denaturation of tropocollagen. In the case of myoglobin, ΔH and Td remained reasonably constant until the protein concentration decreased below 5%, when a modest increase in both Td and ΔH was observed.

3.3 THE STUDY OF THERMAL STABILITY OF CONCANAVALIN A BY DSC

In the current work, the denaturation of Con A at pH 7 was investigated by DSC. The thermogram of Con A is shown in Figure 11. The start of denaturation measured from the DSC thermogram is 70 ^{o}C. The peak temperature of denaturation obtained from the DSC is 81.6 ^{o}C (Fig. 11). The DSC thermogram of Con A at pH 12 is extremely complex, indicating a large number of transitions (Fig. 12).

In some practical studies geared toward the investigation of protein functionality, DSC can be viewed as a secondary analytical technique, since by itself it cannot predict the performance of a specific protein. If information regarding the performance of a specific protein is required, additional physicochemical information is obviously needed. However, the

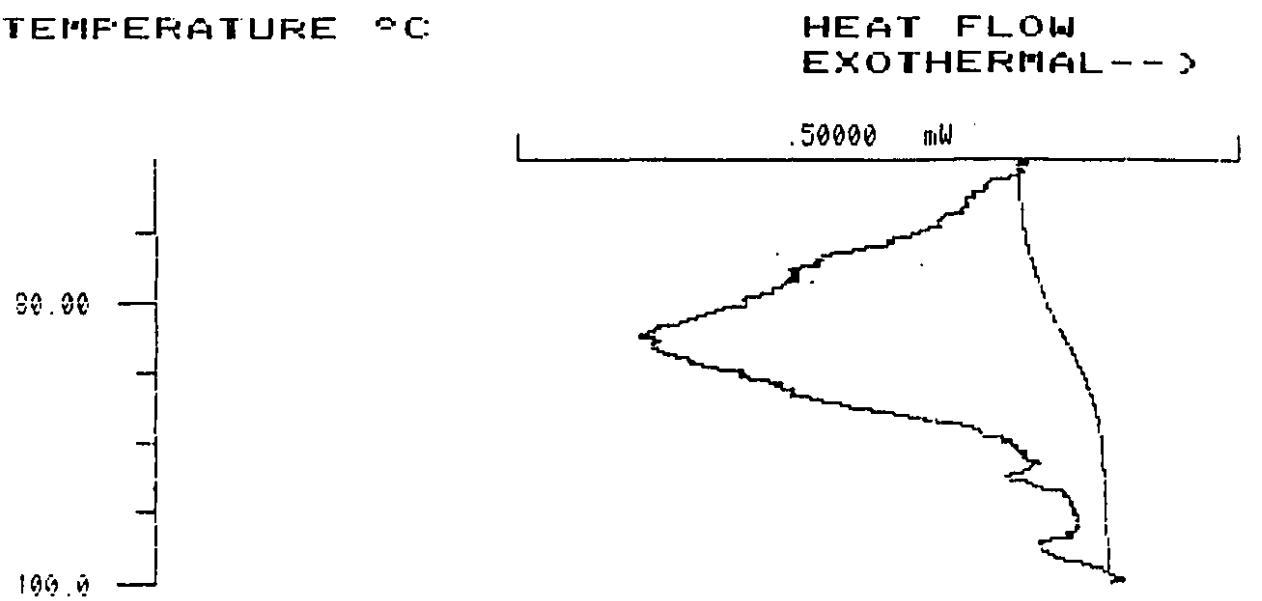

Figure 11 DSC Thermogram of Concanavalin A at pH7

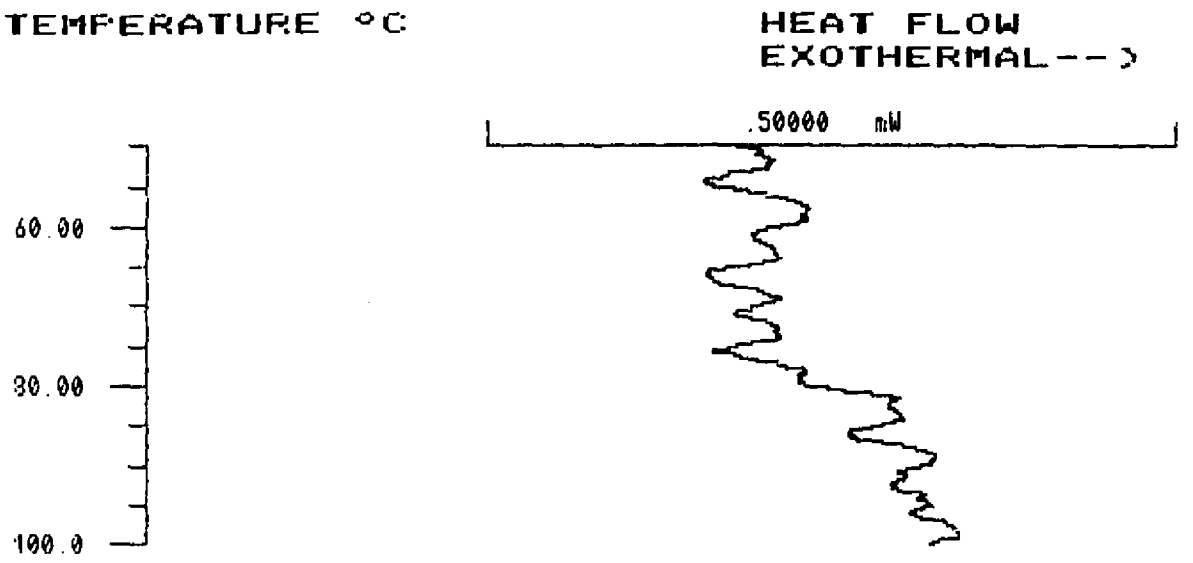

Figure 12. DSC Thermogram of Concanavalin A at pH12

ability of DSC to study processes at the dynamic temperatures occurring during processing, as well as providing thermodynamic and kinetic data, is invaluable to food protein analysis.

CHAPTER 4. THERMAL AND STRUCTURAL ANALYSIS OF CONCANAVALIN A BY FOURIER TRANSFORM INFRARED SPECTROSCOPY

4.1 STUDY OF PROTEIN STRUCTURE AND CONFORMATION BY FTIR SPECTROSCOPY

The three-dimensional structure of proteins can be best determined by X-ray crystallography, although for low-molecular-weight (Mr < 10,000) proteins two-dimensional NMR techniques offer an increasingly viable alternative. However, crystallographic analysis has obvious practical limitations, such as the difficulty of obtaining high-quality protein crystals, the time required for a structure determination and, most importantly, the inherently static character of crystallographic information that cannot be easily extrapolated to the dynamic properties of proteins in solution (Fringeli et al., 1986; Surewicz and Mantsch, 1987). The limitations of X-ray crystallography, along with the growing need to understand the conformational properties of proteins in different environments (e.g., water, membranes, micelles, organic solvents) and under different physiological and non-physiological conditions, have stimulated the search for alternatives to the precise crystallographic determination of the secondary structure of proteins. The spectroscopic techniques currently used for such purposes include NMR, circular dichroism, Raman and infrared spectroscopy.

Infrared spectroscopy was one of the first experimental techniques to be recognized as potentially useful for examination of the secondary

structure of polypeptides and proteins (Elliot & Ambrose, 1950; Susi, 1969; Susi, 1972; Parker, 1983). In the past, the practical usefulness of the method was limited, however, since water absorbs strongly in the most important spectral region for the study of protein conformation, around 1640 cm^{-1}. Thus, studies in aqueous solution were difficult unless deuterium oxide was used as solvent, and the limited sensitivity of infrared instruments made it difficult to extract the structural information contained in the conformation-sensitive infrared bands. The use of FTIR spectrometers to record the infrared spectra of proteins has removed many of the previous limitations, providing improved sensitivity, and allowing more detailed studies of proteins. Another difficulty appears to be of a more fundamental nature. Many studies, both experimental and theoretical, have shown that the conformation-sensitive amide bands of proteins are composites which consist of overlapping component bands originating from different structures, such as α-helices, β-strands, turns and non-ordered polypeptide fragments in proteins. Due to the inherently large widths of these overlapping component bands (greater than the separation between the peak maxima), they cannot be resolved and/or identified in the broad contours of experimentally measured spectra. Thus, until recently the infrared estimates of protein secondary structure were deduced from the global shape of the composite bands (Chirgadze et al., 1973; Rügee et al., 1975; Kotekiansly et al., 1981; Venyaminov et al., 1983; Kleffek et al., 1985; Fringeli et al., 1986).

A significant step forward in the infrared spectroscopic analysis of proteins was made recently by developing computational procedures for the

resolution enhancement of broad infrared bands. These resolution-enhancement techniques such as derivative spectroscopy and Fourier self-deconvolution have been applied to the analysis of protein spectra in order to obtain the positions of the overlapping components of the broad amide bands and assign them to different secondary structure components (Susi & Byler, 1983; Mantsch, et al., 1986; Yang et al., 1985; Thomas et al., 1984; Purcell et al., 1984; Griffiths & de Haseth, 1986).

4.2 THE AMIDE I BAND ASSIGNMENTS AND THE SECONDARY STRUCTURE OF PROTEINS

The amide groups of polypeptides and proteins possess nine characteristic infrared bands (Susi, 1972; Miyazawa, 1956). Many studies have shown that the most structure-sensitive amide band in the infrared spectra of proteins is the amide I band, usually observed between approximately 1600 and 1700 cm^{-1}. The amide I band represents primarily the C=O stretching vibrations of the amide groups coupled to the in-plane NH bending and CN stretching modes (Susi, 1969). The exact frequency of this vibration depends on the nature of hydrogen bonding involving the C=O and NH moieties: this, in turn, is determined by the particular secondary structure adopted by the polypeptide chains (Elliot & Ambrose, 1950; Susi et al., 1967; Timasheff et al., 1967). The observed amide I band contours of proteins are usually complex composites that consist of a number of overlapping component bands, representing helices, β-sheets, turns and non-ordered structures (Itoh & Shimanouchi, 1970; Dwivedi & Krimm, 1984; Sengupta & Krimm, 1985). Attempts have been made to

employ other vibrational bands, such as the amide II band, in the analysis of the polypeptide backbone conformation, but such correlations are still not well established. The amide III mode is also sensitive to the secondary structure, but it gives rise only to very weak infrared bands. Due to vibrational selection rules, the amide III band is much stronger in the corresponding Raman spectra. Thus, Raman spectroscopy seems better suited for the analysis of this vibrational mode (Lavialle et al., 1982; Vincent et al., 1984; Jakobsen et al., 1983; Wasacz et al., 1987; Kaiden et al., 1987) (Table 4).

In order to examine the amide I band, proteins are dissolved in D_2O solution to eliminate the strong interfering absorption by H_2O in the region of interest (Byler & Susi, 1986; Yang et al., 1985; Lee et al., 1985; Olinger et al., 1986; Harris et al., 1986). The application of resolution enhancement techniques has revealed that the amide I band contours can be decomposed into a number of components (Yang et al., 1987; Surewicz et al., 1987; Arrodo et al., 1987). For example, infrared spectra of proteins often exhibit an amide I band component centered between approximately 1650 and 1658 cm^{-1}. These bands are believed to represent α-helical segments (Krimm & Bandekar, 1986). The bands between 1640 and 1648 cm^{-1} are usually associated with a non-ordered polypeptide conformation (Susi, 1969; Parker, 1983; Susi & Byler, 1986). Such bands are observed in the spectra of a number of apparently `orderless' proteins (Byler & Susi, 1986; Surewicz et al., 1987; Purcell & Susi, 1984). However, when a large proportion of amino acids are in an ill-defined `random' conformation, the

TABLE 4. Summary of Frequencies and Assignment of Amide Bands of Proteins and Polypeptides

Band	Usual Frequency Range (cm^{-1})	Approximate description
Amide A	3300	N-H str
Amide B	3100	N-H str (in Fermi resonance with 2 × amide II)
Amide I	1600-1700	C=O str, N-H def, C-N str
Amide II	1480-1575	C-N str, N-H def
Amide III	1229-1301	C-N str, N-H def
Amide IV	625-767	O=C-N def
Amide V	640-800	N-H def
Amide VI	537-606	C=O def
Amide VII	200	C-N def

Data from Susi, 1972; Susi & Byler, 1986; Koenig & Tabb, 1980

amide I band around 1644 cm^{-1} in the deconvolved spectrum is very broad, suggesting that it still represents a composite of a number of closely spaced but unresolved components. In H_2O medium, the amide I mode of amide groups in the random conformation is usually between 1650 and 1660 cm^{-1}, i.e., very close to that corresponding to α-helical structures (Byler & Susi, 1986; Surewicz et al., 1987; Purcell & Susi, 1984).

Infrared bands between 1620 and 1640 cm^{-1} are assigned by many authors to β-strands, although in certain cases β-strands may also have infrared bands at wavenumbers even below 1620 cm^{-1} (Parker, 1983; Susi & Byler, 1986; Krimm & Bandekar, 1986; Byler & Susi, 1986; Surewicz et al, 1987). In addition to these bands, β-strands also have an infrared active mode between approximately 1670 and 1695 cm^{-1} (Krimm & Bandekar, 1986). It has also been proposed to assign bands around 1670, 1683, 1688 and 1694 cm^{-1} to turns. Turns are also associated with a highly characteristic band around 1665 cm^{-1} that has been observed in the spectra of most proteins studied to date (Byler & Susi, 1986; Harris et al., 1986; Yang et al., 1987; Surewicz et al., 1987).

While the correspondence between the most common elements of protein secondary structure and the component amide I bands is relatively well established (Table 5), certain minor or rare structures may interfere with the band assignments discussed above.

TABLE 5. Assignment of Amide I Band Components to Different Types of Protein Conformation

Frequency (cm^{-1})	Assignment	Frequency (cm^{-1})	Assignment
1624±4	Extended chains	1663±4	Turns
1632±2	Extended chains	1669±2	Turns
1638±2	Extended chains	1675±4	Extended chains
1644±2	Unordered	1683±2	β-sheet / Turns
1654±2	α-Helix	1688±2	β-sheet / Turns
		1694±2	β-sheet / Turns

Data from Byler & Susi, 1986; Yang et al., 1987; Surewicz et al., 1987.

4.3 EXPERIMENTAL

Con A was obtained from Sigma and used as received. Solutions of Con A in D_2O with varying concentrations were prepared for studies of the effect of concentration. For the effect of pH, phosphate buffers prepared in D_2O (PBS/D_2O) were used. Infrared spectra at 4 cm^{-1} resolution were recorded with a Nicolet 8210E FTIR spectrometer. The samples were held in temperature controlled cells of 50 μm pathlength fitted with CaF_2 windows. 512 scans were co-added and the resulting spectra were deconvolved using K = 2 and band width of 13. For the pressure studies, spectra were recorded with a Nicolet 510 spectrometer equiped with a liquid-nitrogen cooled mercury cadmium telluride detector. Samples were placed in a diamond anvil cell together with a very small amount of quartz powder. Pressures at the sample were determined from the 695-cm^{-1} infrared absorption of α-quartz, as described by Wong et al. (1985).

4.4 STRUCTURAL ANALYSIS OF CON A BY FTIR

The infrared spectrum of a 10% solution of concanavalin A (pH 7) was recorded at room temperature using phosphate buffer (prepared in D_2O) as solvent. After Fourier self-deconvolution of the amide I' band, several bands were observed (Fig. 13). The assignments of these bands have previously been reported by Casal et al. (1988). The most intense band, located at 1635 cm^{-1}, and a shoulder at 1683 cm^{-1} were assigned to antiparallel ß-sheet. The shoulders at 1645 and 1653 cm^{-1} may be assigned to unordered (random coil) and α-helical structures, respectively. The band

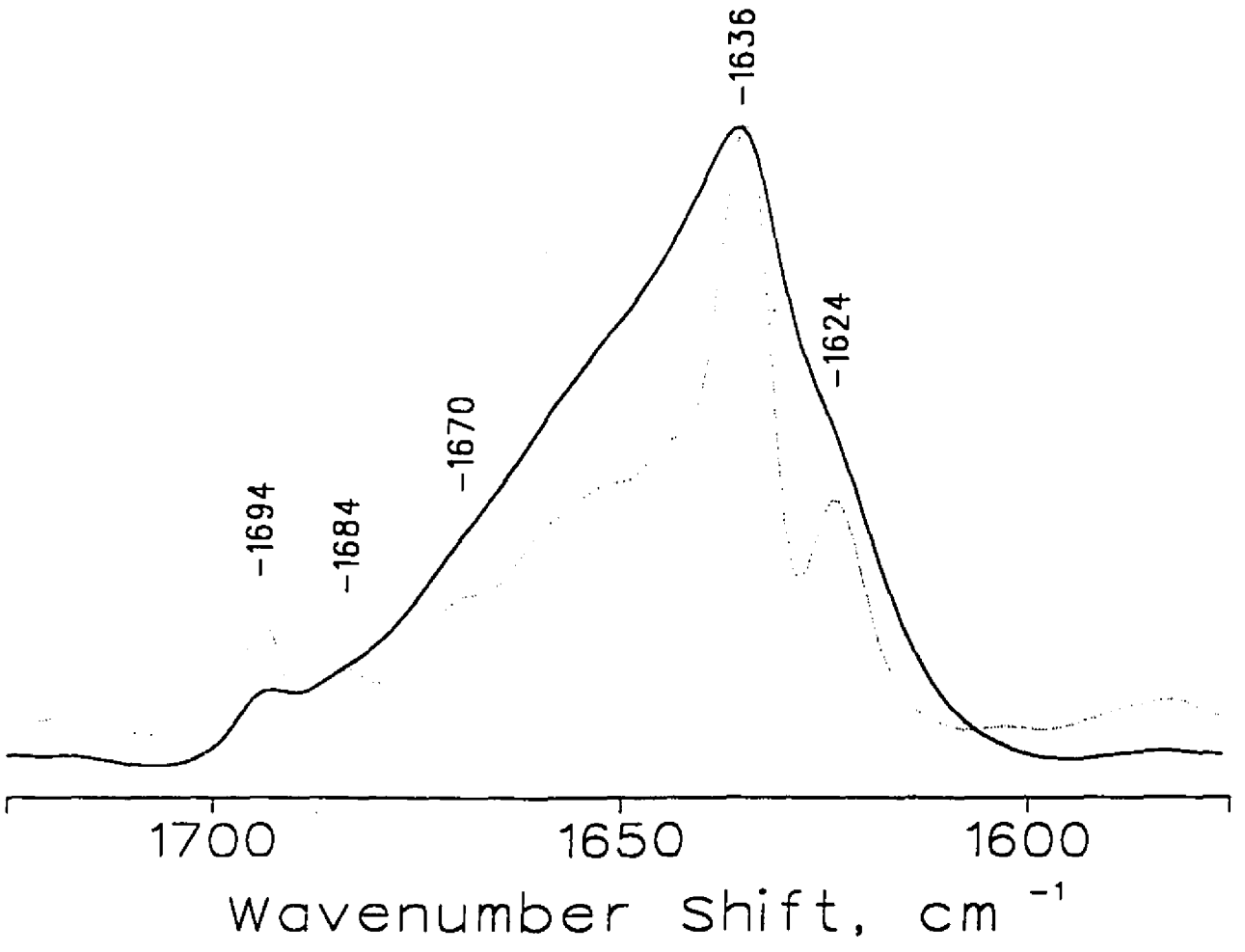

Figure 13a (-) The Amide I' Band in the Infrared Spectrum of 10% Concanavalin A before Self-deconvolution

Figure 13b (.) The Amide I' Band in the Infrared Spectrum of 10% Concanavalin A after Self-deconvolution

at 1624 cm^{-1} was attributed to `exposed' β-strands, i.e., to those β-strands that are not part of the core of the β-sheet. The band at 1694 cm^{-1} was assigned to protonated carboxylate groups by Casal et al. (1988), but in 1989, Byler et al. reported that bands near 1690 cm^{-1} are usually due to turns, although one cannot absolutely rule out possible contributions by the high frequency components of β-strands.

4.4.1 Effect of Concentration

The infrared spectra of concanavalin A at various concentrations in phosphate buffer (prepared in D_2O) were studied at room temperature. Fig. 14 shows the amide I' band in the infrared spectra of concanavalin A before and after self-deconvolution at concentrations ranging from 1 to 30% (w/v). Examination of these spectra reveals that there are no differences in the secondary structure of Con A associated with changes in protein concentration in the range from 1 to 30% at room temperature.

4.4.2 Effect of pH

It is known that some proteins undergo several changes as a function of pH. These are related to aggregation and disaggregation which, at least in certain cases, are thought to be accompanied by conformational and/or structural changes.

Changes in the infrared spectrum of Con A as a function of changes in pH were investigated in order to detect and characterize conformational

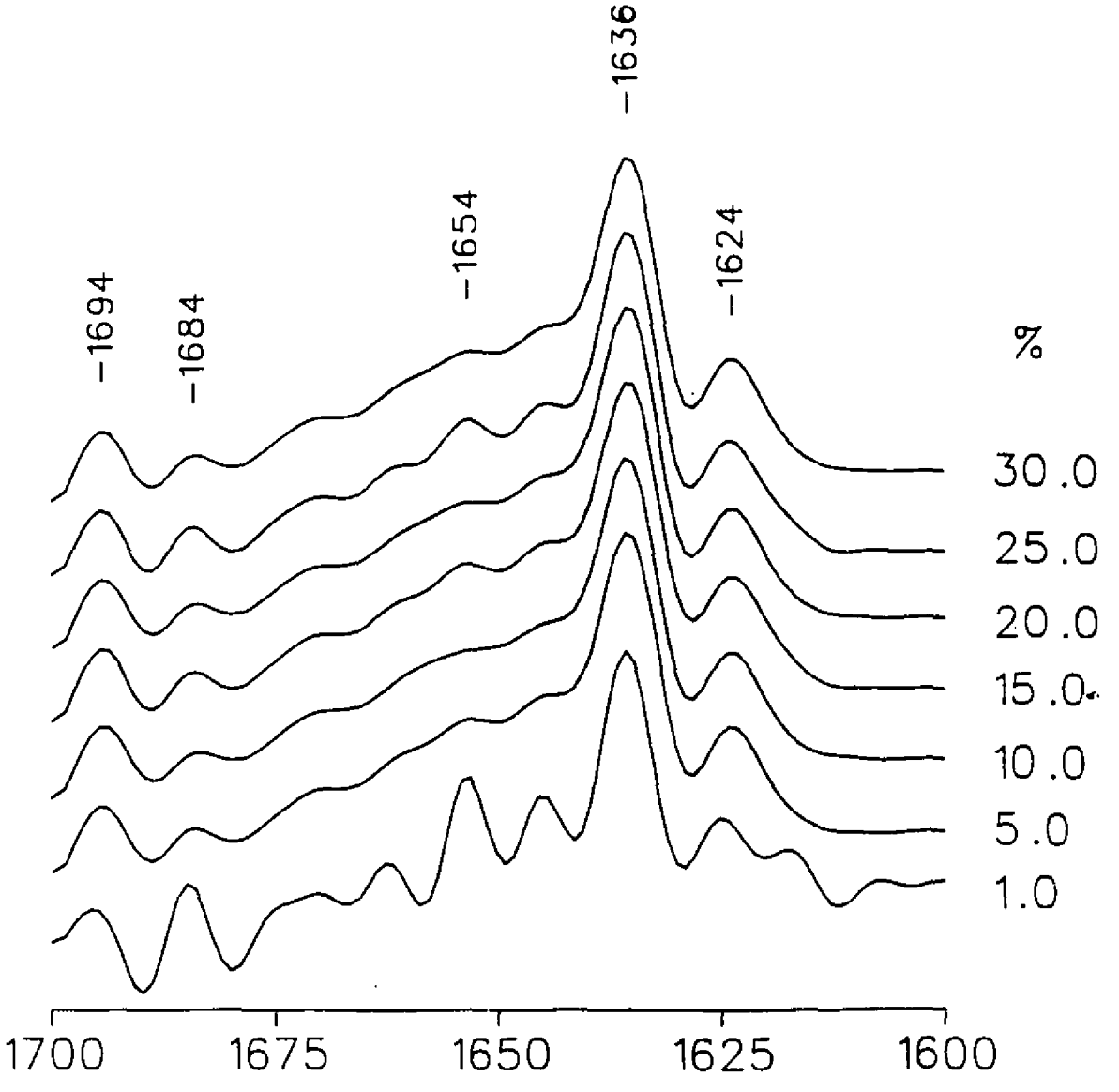

Figure 14 The Amide I' Band in the Infrared Spectra of Concanavalin A at Various Concentrations

changes. Fig.15 shows the IR spectra of Con A at various pHs at room temperature. From these spectra, it can be seen that between pH 2 and pH 10, there are hardly any differences in the IR spectra, except for a small increase in the intensity of the 1644 cm^{-1} band which is attributed to unordered structure as mentioned previously, and a small decrease in the intensity of the 1624 cm^{-1} band with increasing pH. This indicates that in acidic and midly basic regions of the pH scale there is practically no change in the secondary structure of Con A. At pH 12, a substantial change in the IR spectrum occurs. The bands at 1694 cm^{-1}, 1624 cm^{-1} and 1635 cm^{-1} become very broad, and two new bands appear at 1614 cm^{-1} and 1684 cm^{-1}. In an FTIR study of the denaturation of chymotrypsinogen, the appearance of bands at 1684 and 1618 cm^{-1} was attributed to the formation of intermolecular β-sheet structure. Thus, these bands are associated with protein aggregation.

4.4.3 Effect of Sugars

Fig.16 shows the amide I' band in the infrared spectra of Con A in the presence of different sugars at room temperature. No obvious difference in the amide I band is observed in the presence of the different sugars. This indicates that there are no major changes in the secondary structure of the protein with binding of these sugars (arabinose, galactose, glucose, lactose, maltose, mannose, xylose) at room temperature.

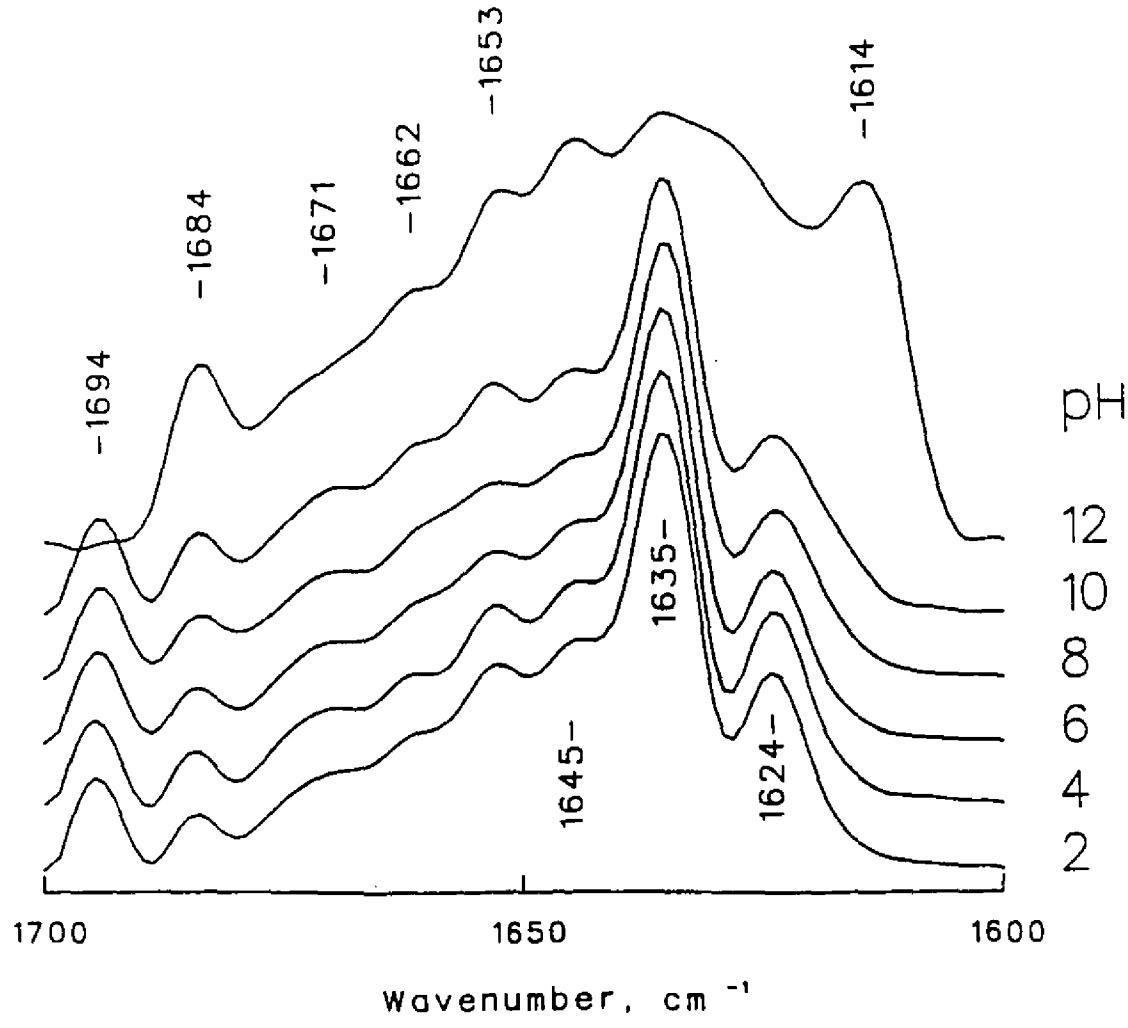

Figure 15 The Amide I' Band in the Infrared Spectra of Concanavalin A at Various pHs

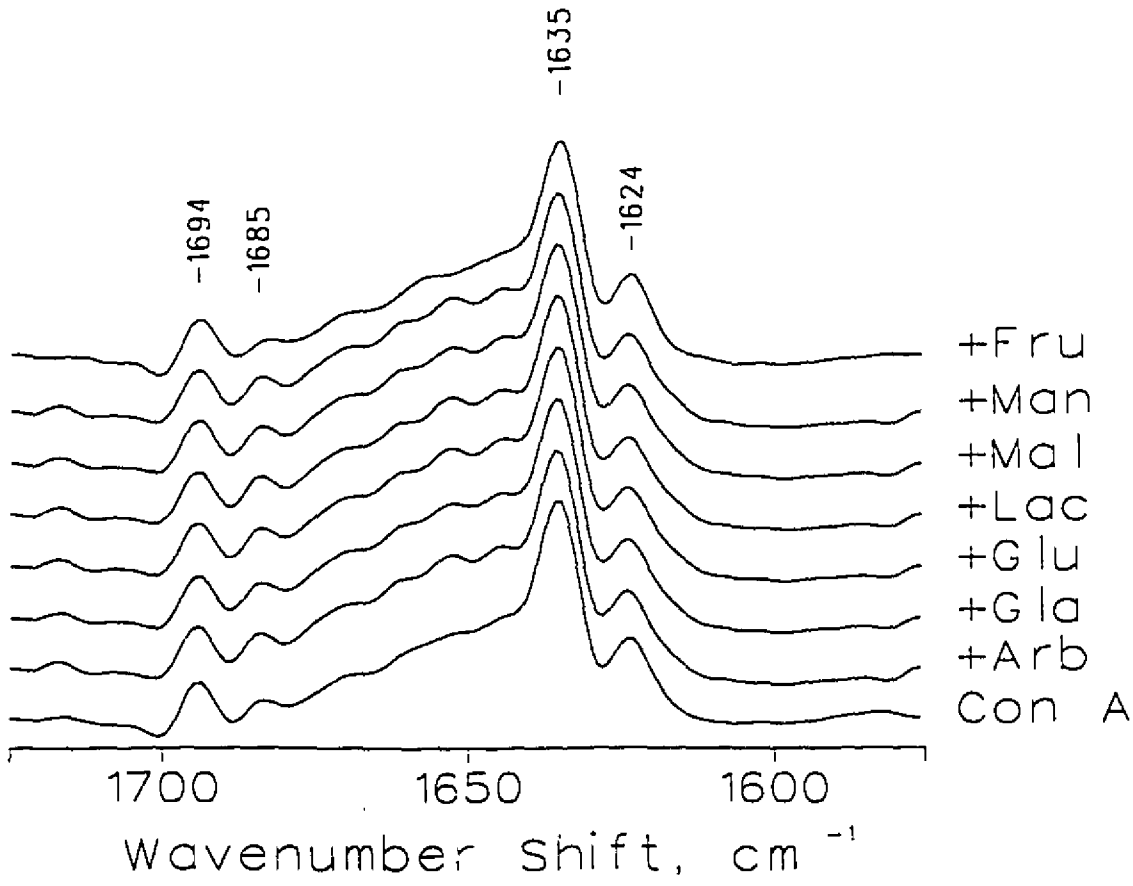

Figure 16 The Amide I' Band in the Infrared Spectra of Concanavalin A in the Presence of Different Sugars

4.4.4 Effect of Metal Ions

Fig.17 shows the amide I' band in the infrared spectra of concanavalin A in the presence of several kinds of metal ions. No obvious difference among these spectra is observed, indicating that these ions do not affect the secondary structure of concanavalin A at pH 7 at ambient temperature.

4.4.5 Effect of Temperature

It is well known that protein denaturation may be induced by temperature. In recent years, FTIR spectroscopy has proved very useful in monitoring structural and conformational changes of proteins as a function of temperature (Casal et al., 1988; Ismail, et al., 1992).

Figures 18 and 19 show stacked plots of the deconvoluted amide I' band in the infrared apectra of Con A with increasing temperature from 25.9 to 96.6°C. Very little change in the amide I' band is observed between 25 and 76°C. At 80°C, two new bands are observed at approximately 1618 cm^{-1} and 1683 cm^{-1}, and these bands continue to increase with increasing temperature, with concomitant blurring of the Con A amide I' bands. The appearance of these bands was also accompanied by gel formation in the IR cell. The 1618 and 1683 cm^{-1} bands have been previously assigned in a variable-temprature study of chymotrysinogen (Ismail et al., 1992) to the formation of intermolecular anti-parallel β-sheet. Thus, the unfolding of the protein is accompanied by rapid aggregation at 80 °C.

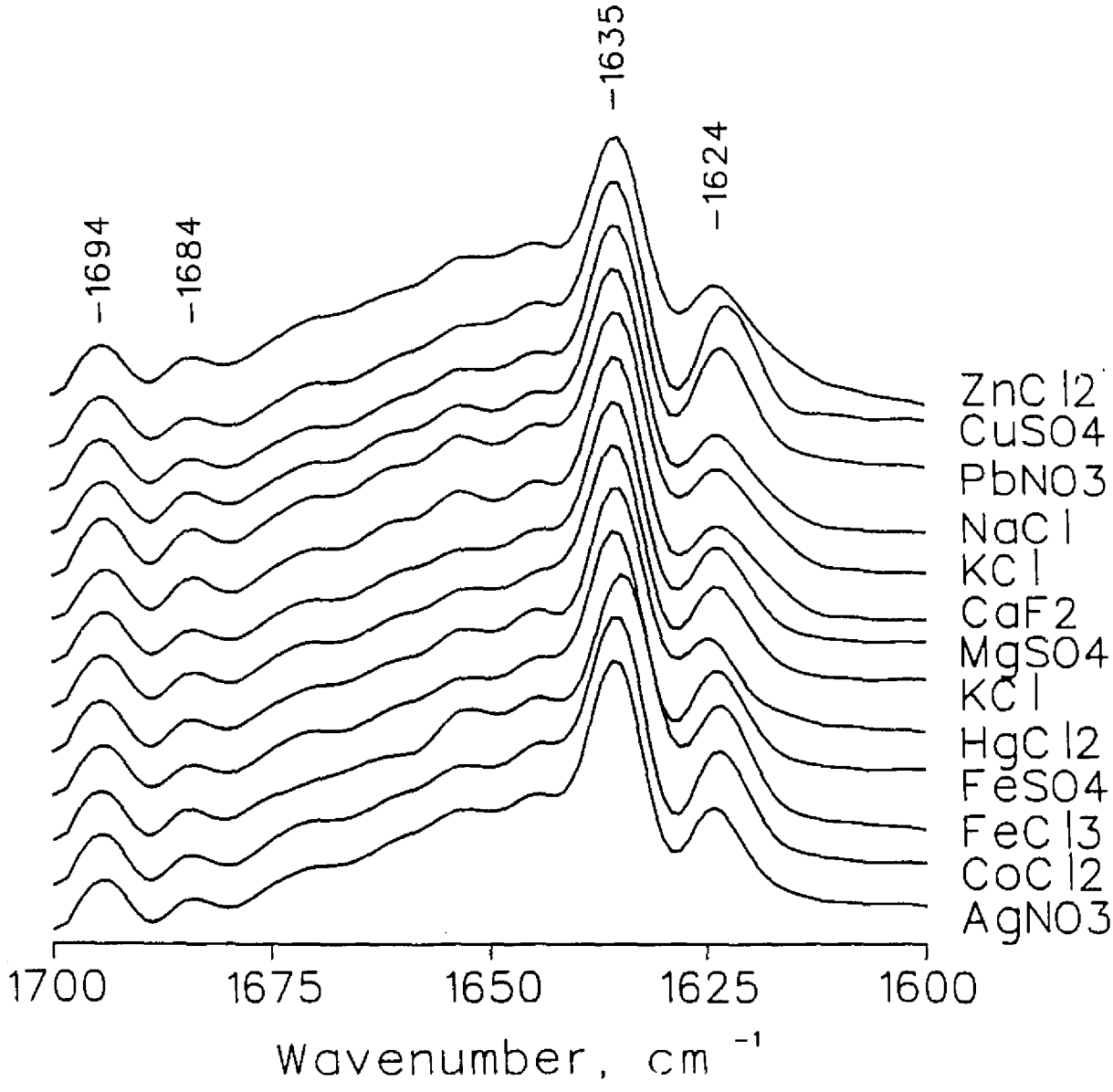

Figure 17 The Amide I' Band in the Infrared Spectra of Concanavalin A in the Presence of Several Kinds of Metal Ions

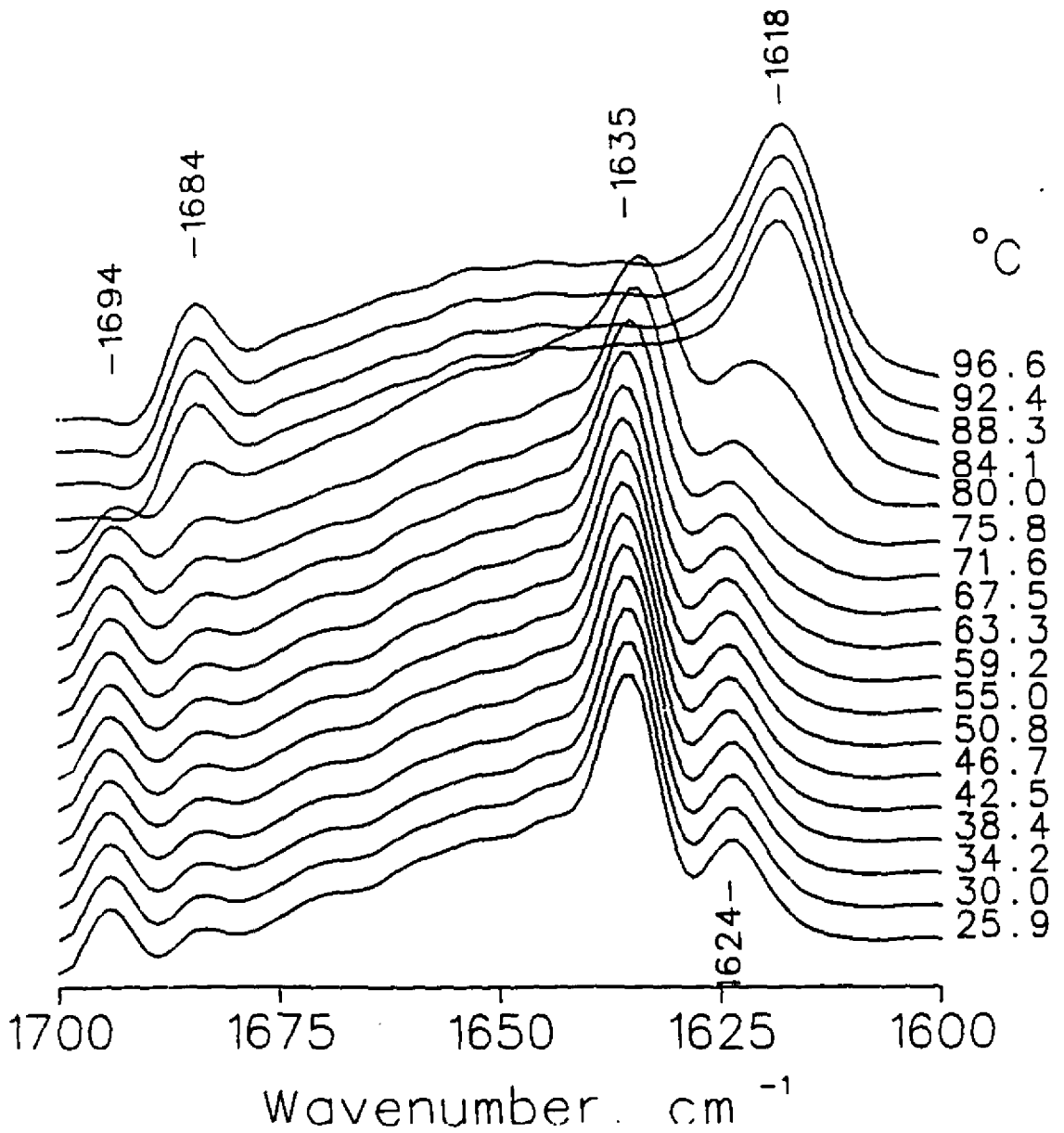

Figure 18 Stacked Plot of the Deconvoluted Amide I' Band of Concanavalin A with Increasing Temperature from 25.9 to 96.6 °C

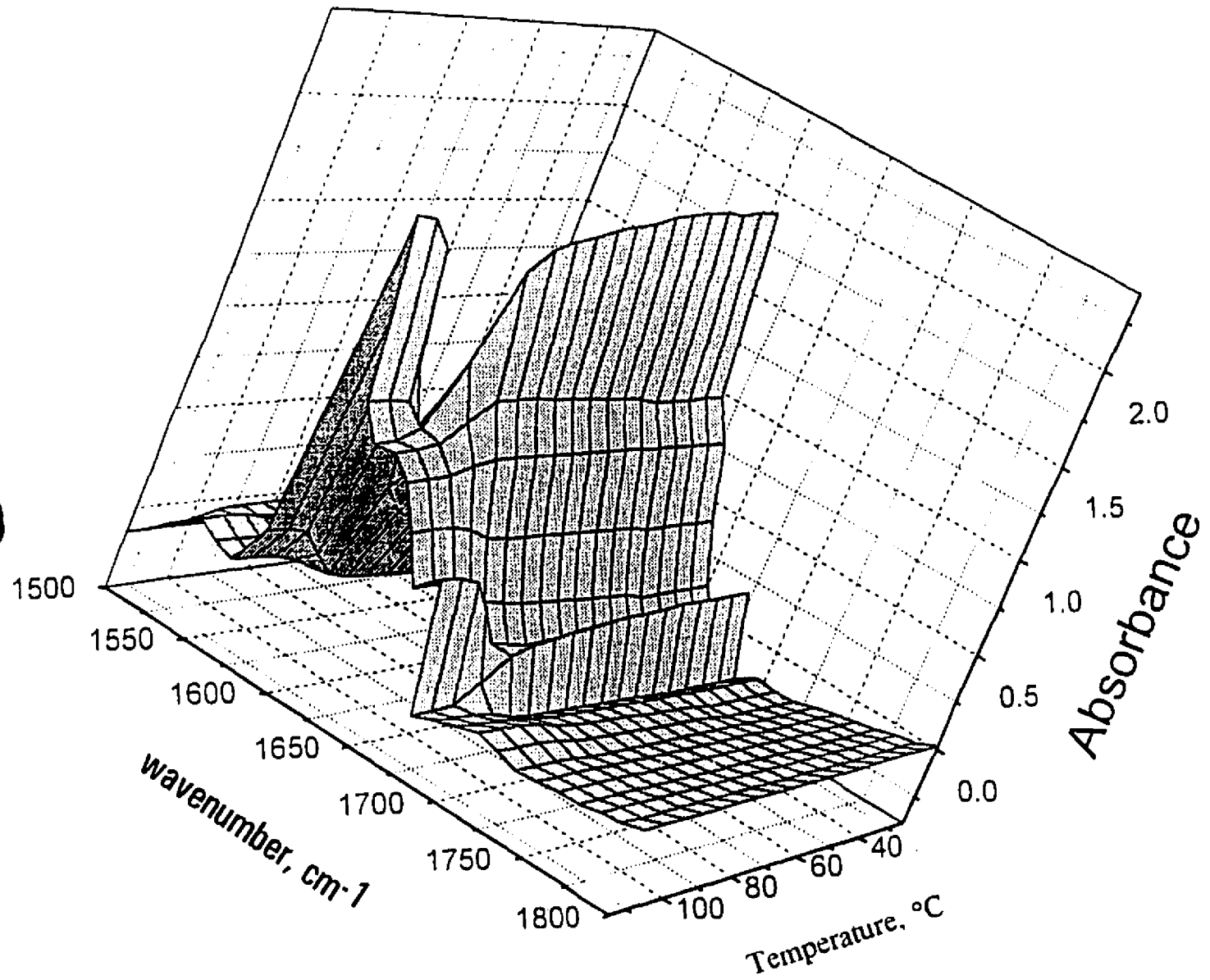

Figure 19 Three-Dimensional Plot of the Deconvoluted Amide I' Band of Concanavalin A as a Function of Temperature

Thus, heating a solution of Con A from about 26 °C to 97 °C results in an increase in the half-width of the amide I band with a transition temperature at 80 °C (Figs. 18 and 19). The 1694 cm^{-1} band disappears at the transition temperature. Between 80 and 84 °C the protein loses most of its intramolecular β-sheet structures with an accompanying appearance of new bands at 1618 and 1684 cm^{-1}, assigend to intermolecular β-sheet stucture.

4.4.6 Effect of Pressure

One of the characteristics of proteins is that their native conformation can be converted to the denatured state by changing the chemical or physical conditions of the medium. The study of pressure-induced denaturation, in particular, has gained considerable interest. Pressure variations at constant temperature are particularly informative because they allow us to focus on volume effects without imparting any thermal energy to the system, contrary to the more common temperature-induced denaturation. A number of experimental techniques have been used to study pressure-induced protein denaturation such as ultraviolet, visible, fluorescence and Raman spectroscopy. Recently, FTIR spectroscopy in combination with a high pressure diamond anvil cell has been employed to study the effect of presence on a variety of proteins. (Wong et al., 1988).

Fig. 20 shows the IR spectra of a 10% solution of Con A in D_2O at various pressures in the range 0 - 14.4 kbar at room temperature. As the external pressure is increased, there is a slight increase in the intensities of

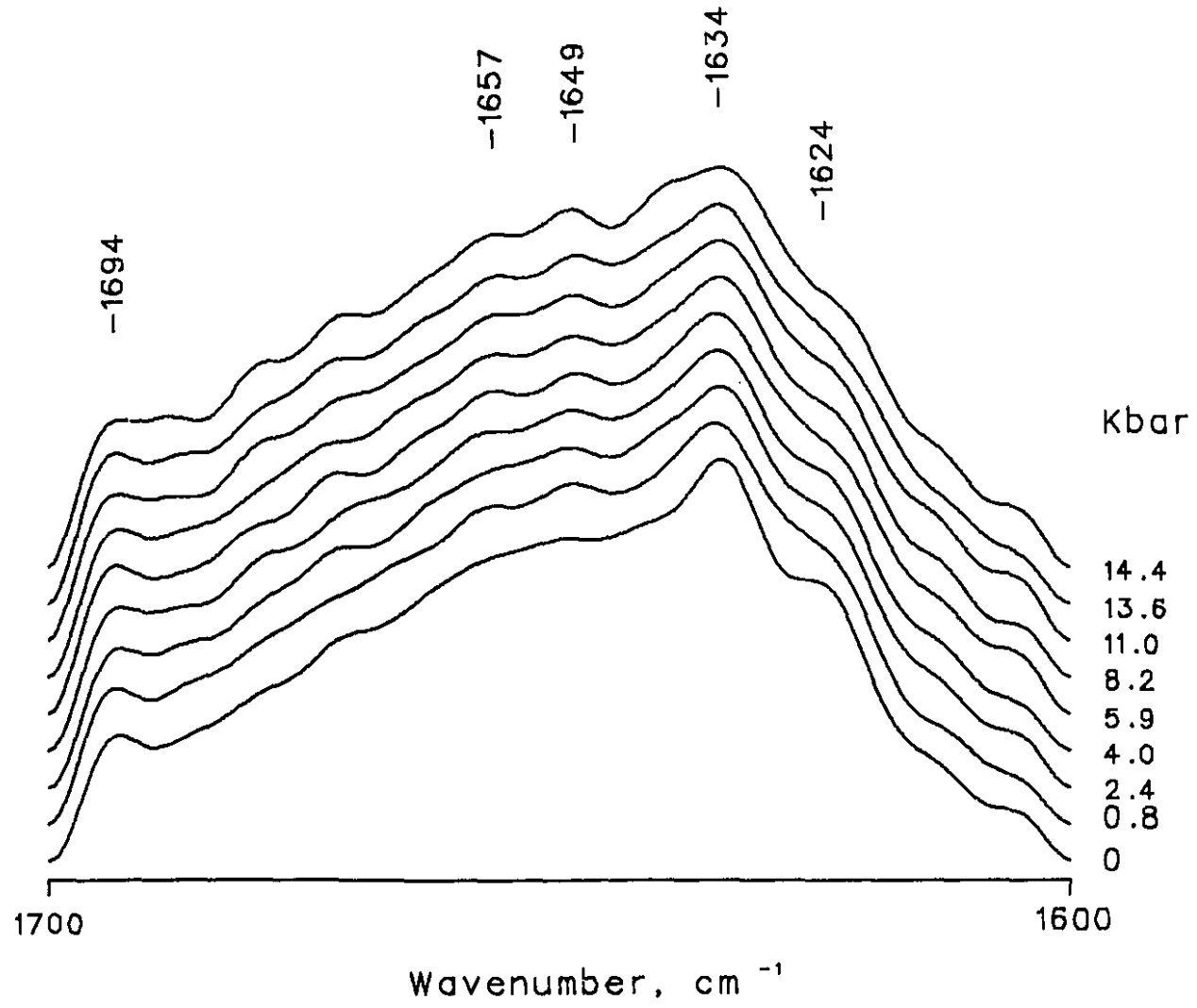

Figure 20 Amide I' Band in the Infrared Spectra of Concanavalin A under Various Pressures

the α-helix band at 1657 cm^{-1} and the band at 1649 cm^{-1} and a decrease of the bands at 1624 and 1634 cm^{-1}. However, no major changes in the infrared spectrum are observed in the pressure range 0 - 14.4 kbar, indicating that Con A does not undergo denaturation in this pressure range.

4.5 VARIABLE-TEMPERATURE FTIR STUDIES OF CON A AS FUNCTION OF CHANGES IN PHYSICO-CHEMICAL PARAMETERS

4.5.1 *FTIR spectroscopy.*

Figures 21-26 show the temperature profiles of the amide I band in the infrared spectrum of concanavalin A heated from 25 to 97°C in 5°C increments at various pHs. At pH 2 (Fig. 21), the 1694 cm^{-1} band disappeared at about 71.6°C along with the band at 1635 cm^{-1}. At the same temperature, the aggregation bands at 1617 and 1684 cm^{-1} appeared. At pH 4 and pH 6 (Figs. 22 and 23), it can be seen that the aggregation bands appeared at about 75.8 °C. This is about 4 °C higher than at pH 2, indicating that at pH 4 and pH 6, Con A is more stable than at pH 2. When Con A was heated at pH 8 and pH 10 (Figs. 24 and 25), bands at 1616 and 1684 cm^{-1} appeared at about 80 °C, and the most intense band at 1635 cm^{-1} and the band at 1694 cm^{-1} completely disappeared when the temperature reached about 85°C, which is about 5°C higher than the

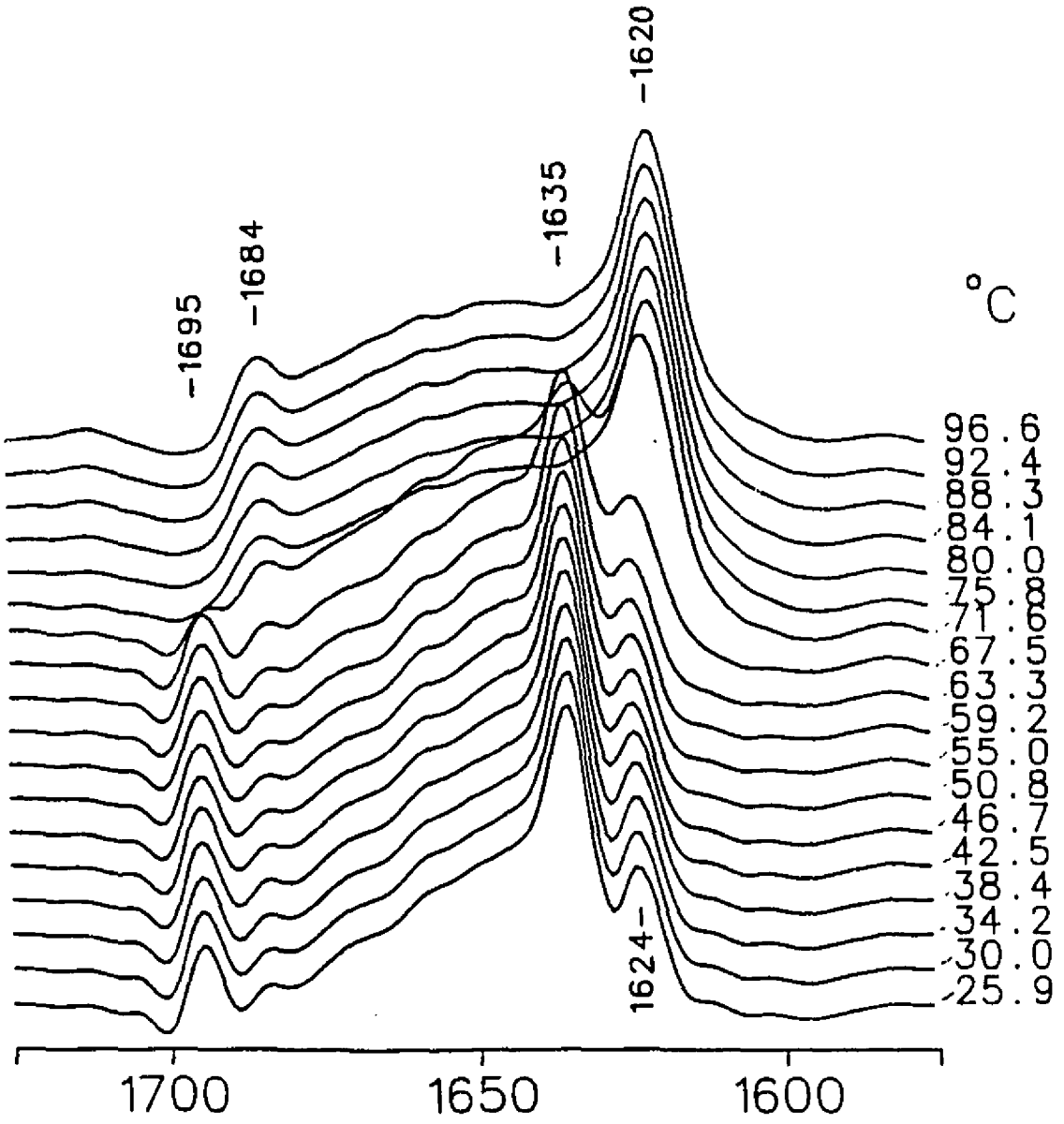

Figure 21 Temperature Profiles of the Amide I' Band of Concanavalin A Heated from 25 to 97 °C in 5 °C Increments at pH 2

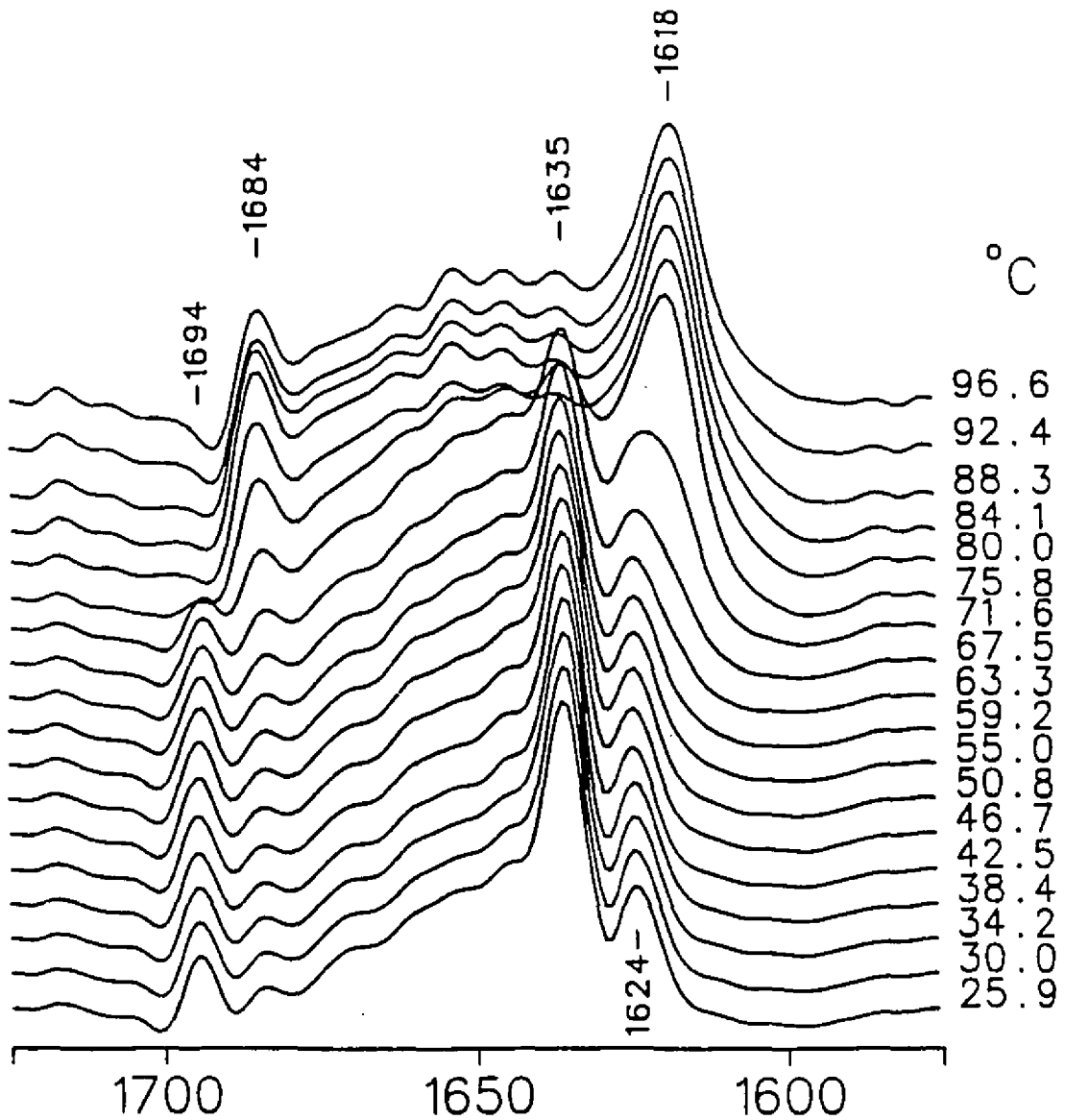

Figure 22 Temperature Profiles of the Amide I' Band of Concanavalin A Heated from 25 to 97 °C in 5 °C Increments at pH 4

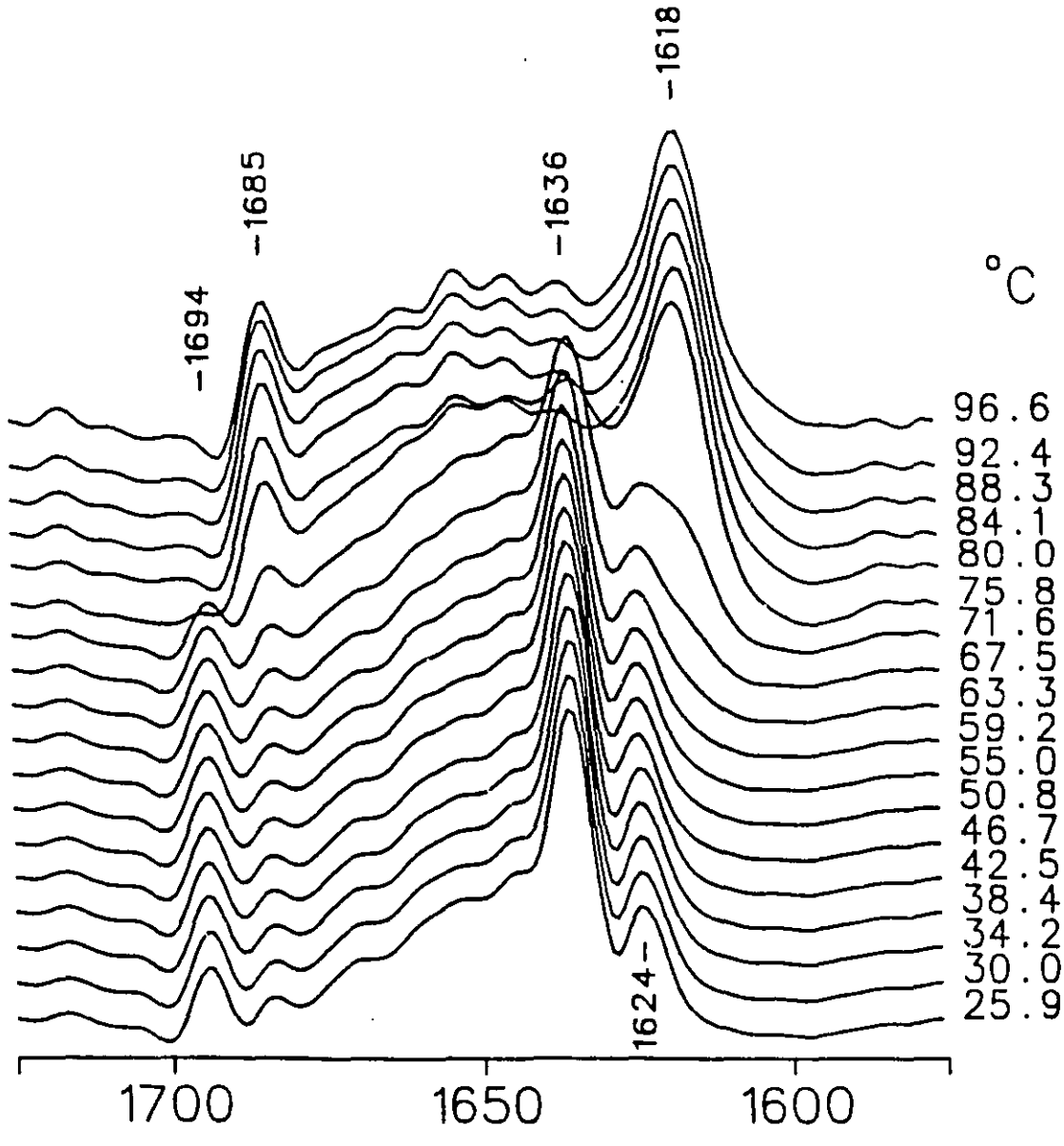

Figure 23 Temperature Profiles of the Amide I' Band of Concanavalin A Heated from 25 to 97 °C in 5 °C Increments at pH 6

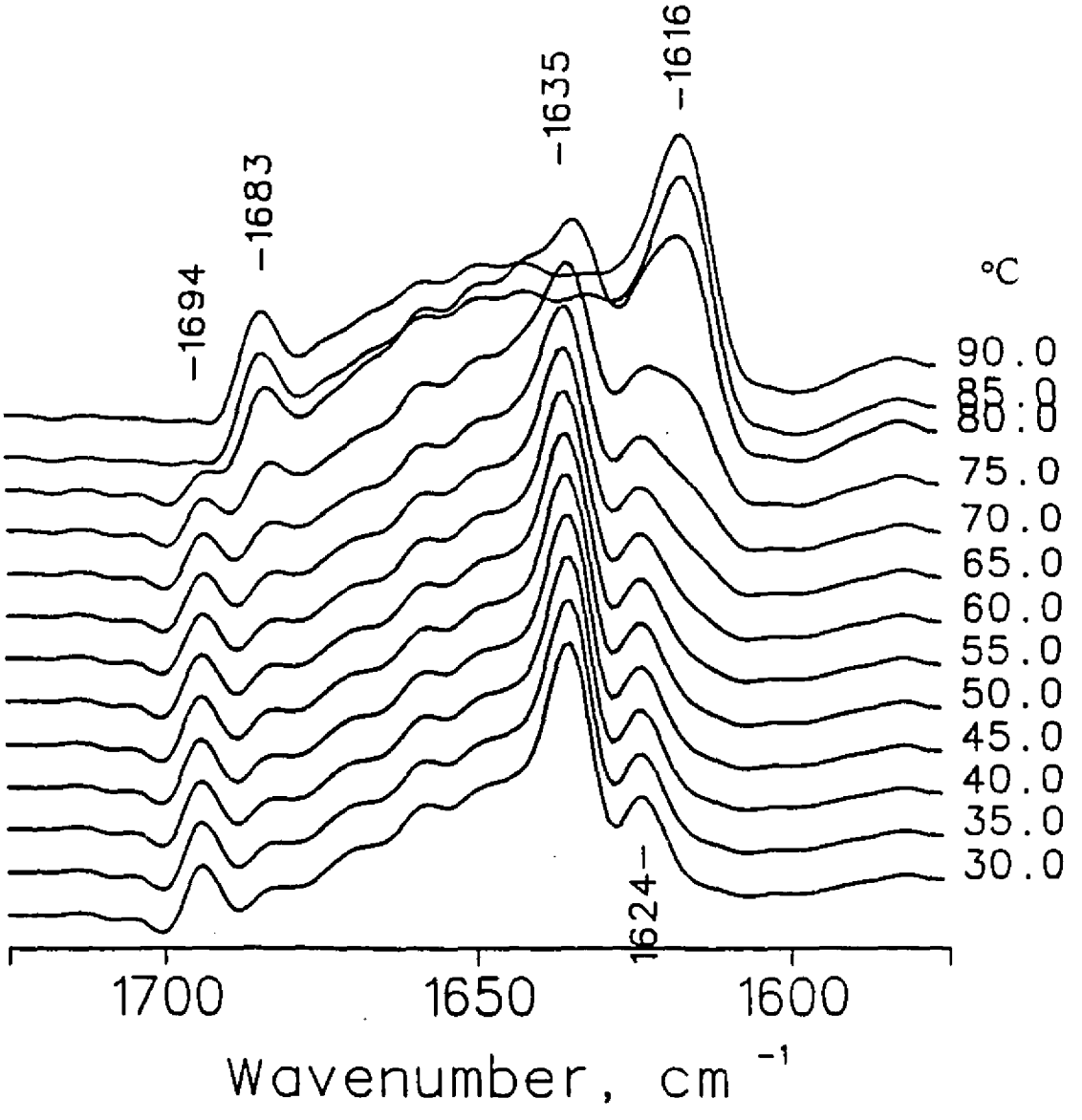

Figure 24 Temperature Profiles of the Amide I' Band of Concanavalin A Heated from 30 to 90°C in 5 °C Increments at pH 8

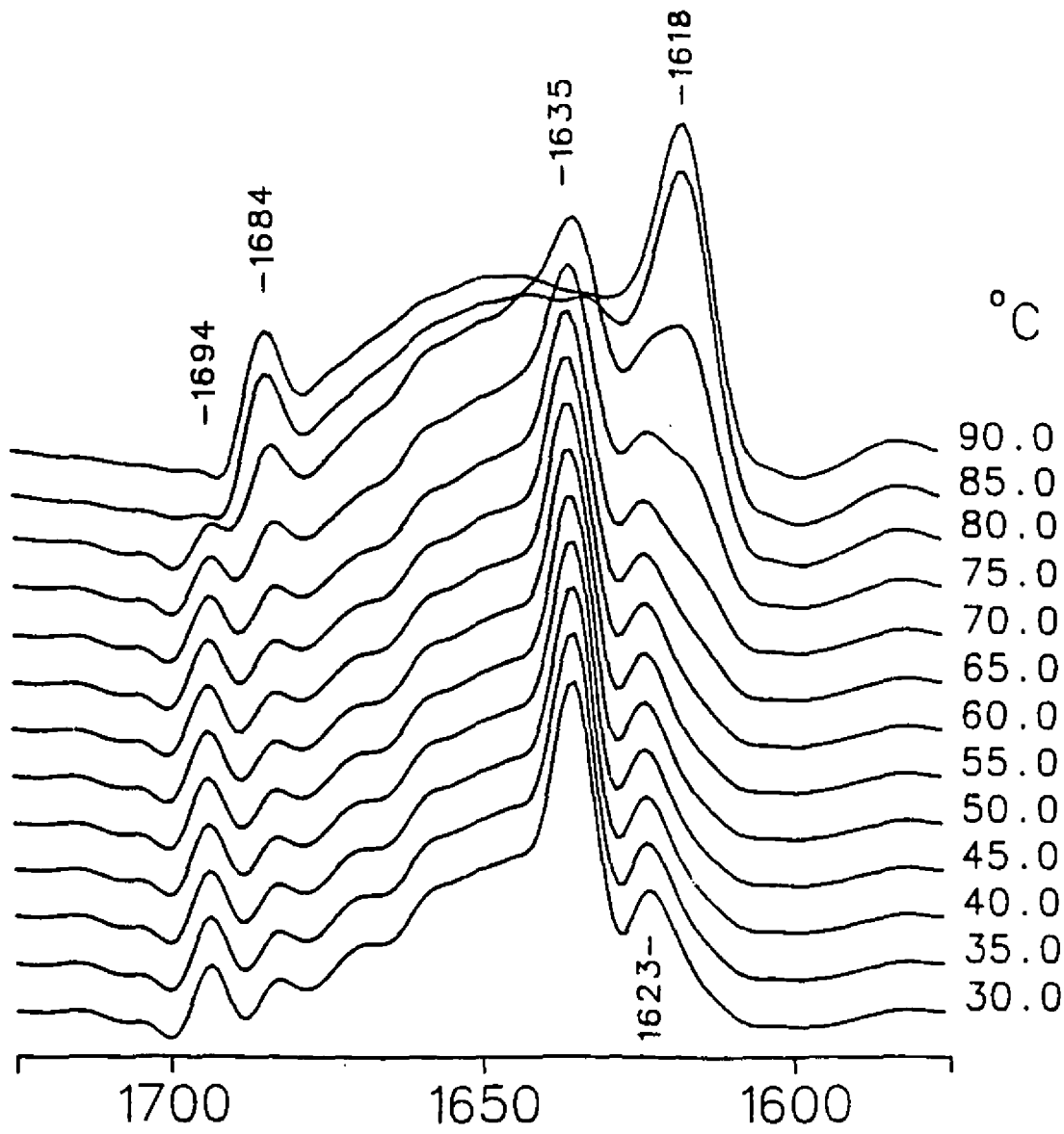

Figure 25 Temperature Profiles of the Amide I' Band of Concanavalin A Heated from 30 to 90°C in 5 °C Increments at pH 10

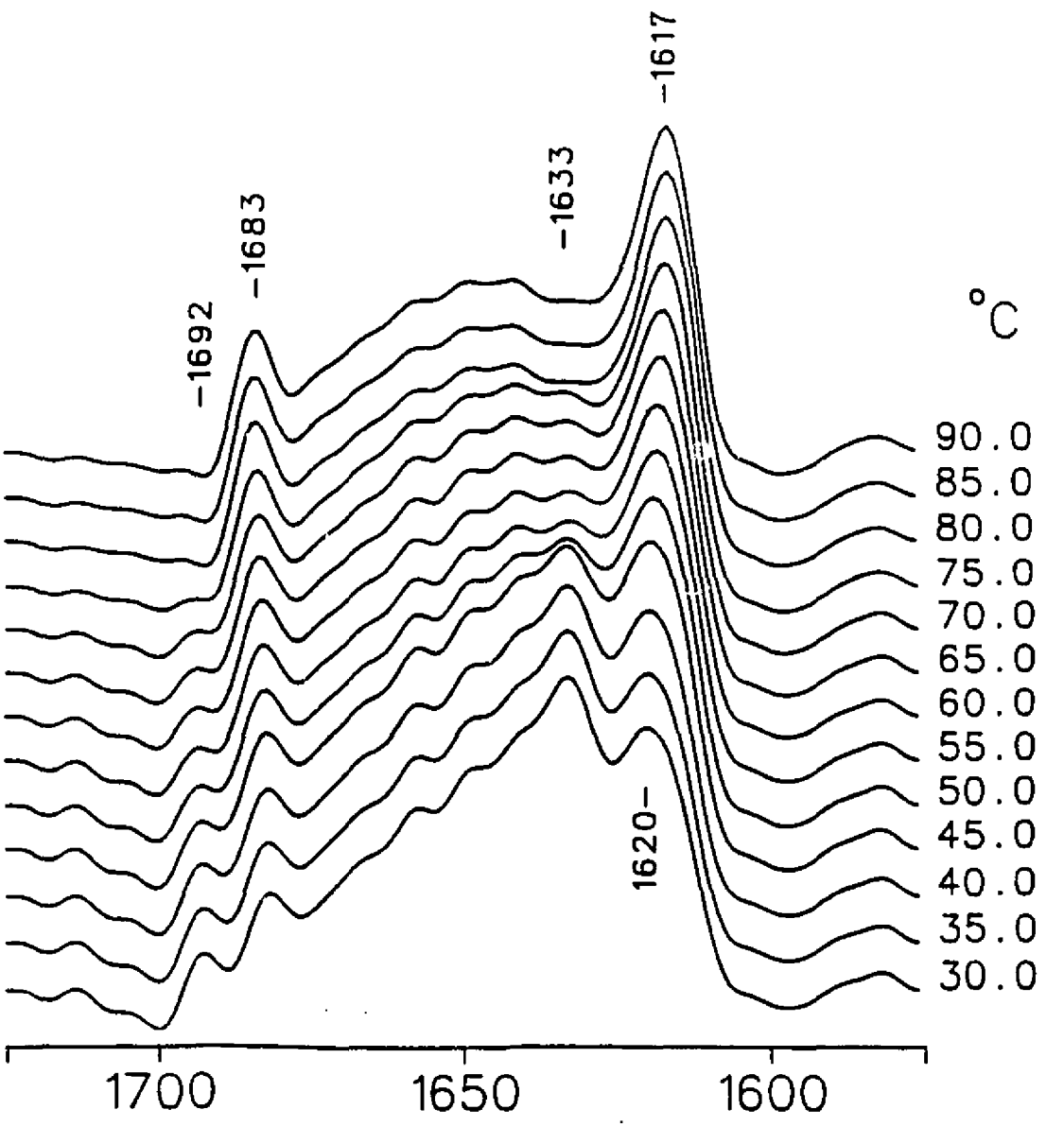

Figure 26 Temperature Profiles of the Amide I' Band of Concanavalin A Heated from 30 to 90 °C in 5 °C Increments at pH 12

temperature at which the aggregation band at 1616 cm^{-1} first appeared. These results show that Con A is more stable at pH 8 - 10 than under acidic or neutral conditions. The spectrum obtained at pH 12 (Fig. 26) shows that under highly alkaline conditions, Con A is least stable. The band at 1633 cm^{-1} almost disappears at approximately 50 °C. The band at 1692 cm^{-1} decreases in intensity with increasing temperature until it totally disappears at 80°C. The intensities of the bands at 1683 cm^{-1} and 1620 cm^{-1} (which gradually shifts to 1617 cm^{-1}) increase with heating. The results of this examination of the thermal stability of concanavalin A at various pHs are summarized in Table 6.

4.5.2 VT-FT Studies of Con A in the Presence of Monosaccharides

Previous studies have demonstrated that very little change in the secondary structure of Con A occurs upon binding of mannose or other sugars (Section 4.4.3). Fig. 27 shows the amide I' band in the FTIR spectrum of 10% Con A heated in the presence of mannose (10% w/v) at pH 7. Examination of the stacked plot in Fig. 27 reveals that the aggregation band at 1617 cm^{-1} appears at a temperature of about 84.1°C. This is about 10°C higher than in the absence of mannose. This may indicate an increase in the propensity of Con A to resist denaturation as well as aggregate formation upon binding to mannose, or it may result from the effect of the sugar reducing the water activity of the solution. The presence of mannose in solution results in retention of some of the secondary structure of Con A even at 95°C. These results show that the sugar imparts significant stability against unfolding under extreme thermal stress.

TABLE 6. The Thermal Stability of Con A as a Function of pH as Monitored by Changes in the Amide I' Band Profile

pH	1618 cm^{-1} (O) °C	1624 cm^{-1} (L) °C	1635 cm^{-1} (L) °C	1684 cm^{-1} (O) °C	1695 cm^{-1} (L) °C
2	71.6	71.6	75.8	⇑	75.8
4	75.8	75.8	80.0	⇑	80.0
6	75.8	75.8	80.0		80.0
8	80.0	80.0	85.0	⇑	85.0
10	80.0	80.0	85.0	⇑	85.0
12	30.0	30.0	55.0	⇑	70.0

(O) Temperature at which band appears. (L) Temperature at which band completely disappears.

(⇑) Increase in band intensity with increasing temperature.

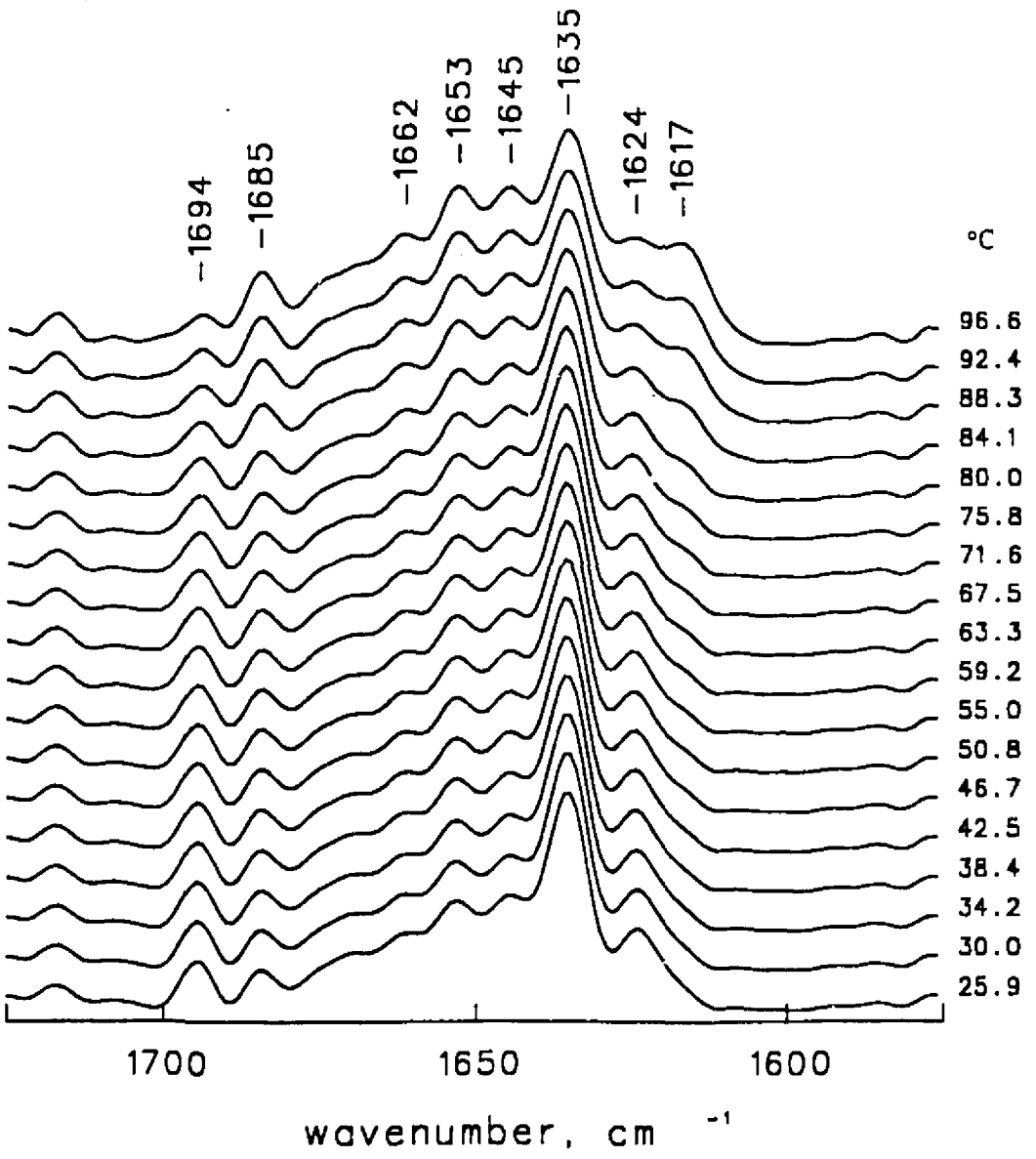

Figure 27 Amide I' Band in the FTIR Spectrum of Concanavalin A Heated in the Presence of 10% Mannose

In order to ascertain whether the stabilizing effect of mannose is due to its high affinity in binding to Con A, the above experiment was repeated with various other sugars in place of mannose. Figure 28 shows the amide I' band in the spectra of a solution containing 10% Con A and 10% fructose versus temperature. Examination of the amide I' band reveals that, even upon heating Con A to 96°C, no change in the amide I' band is observed, indicating that the secondary structure of Con A remains remarkably intact. Examination of the region between 1750 and 1700 cm^{-1} shows that a band centered at 1729 cm^{-1} grows in intensity with increasing temperature. A band at 1729 cm^{-1} has been recently assigned to the open-chain form of fructose, and variable temperature FTIR spectroscopy demonstrated that the equilibrium between the closed- and open-chain form of fructose is shifted in favour of the open-chain form with increasing temperature (Yaylayan & Ismail, 1992). Accordingly, the band observed at 1729 cm^{-1} in Fig. 28 can be assigned to the open-chain form of fructose.

Heating Con A in the presence of sucrose or maltose in the same concentration range (10% w/v) results in very little protection of Con A against denaturation, as indicated by the appearance of the aggregation bands at 1620 and 1683 cm^{-1} with blurring of the amide I' band profile of the native Con A (Fig. 29). These results appear to suggest that water activity is not the key factor in protection of Con A against thermal denaturation. Glucose, which has some affinity for the binding site of Con A, was also found to provide little protection against thermal denaturation of Con A. In the presence of galactose (see Fig. 30), no protection was observed in the temperature range from about 25 to 100°C .

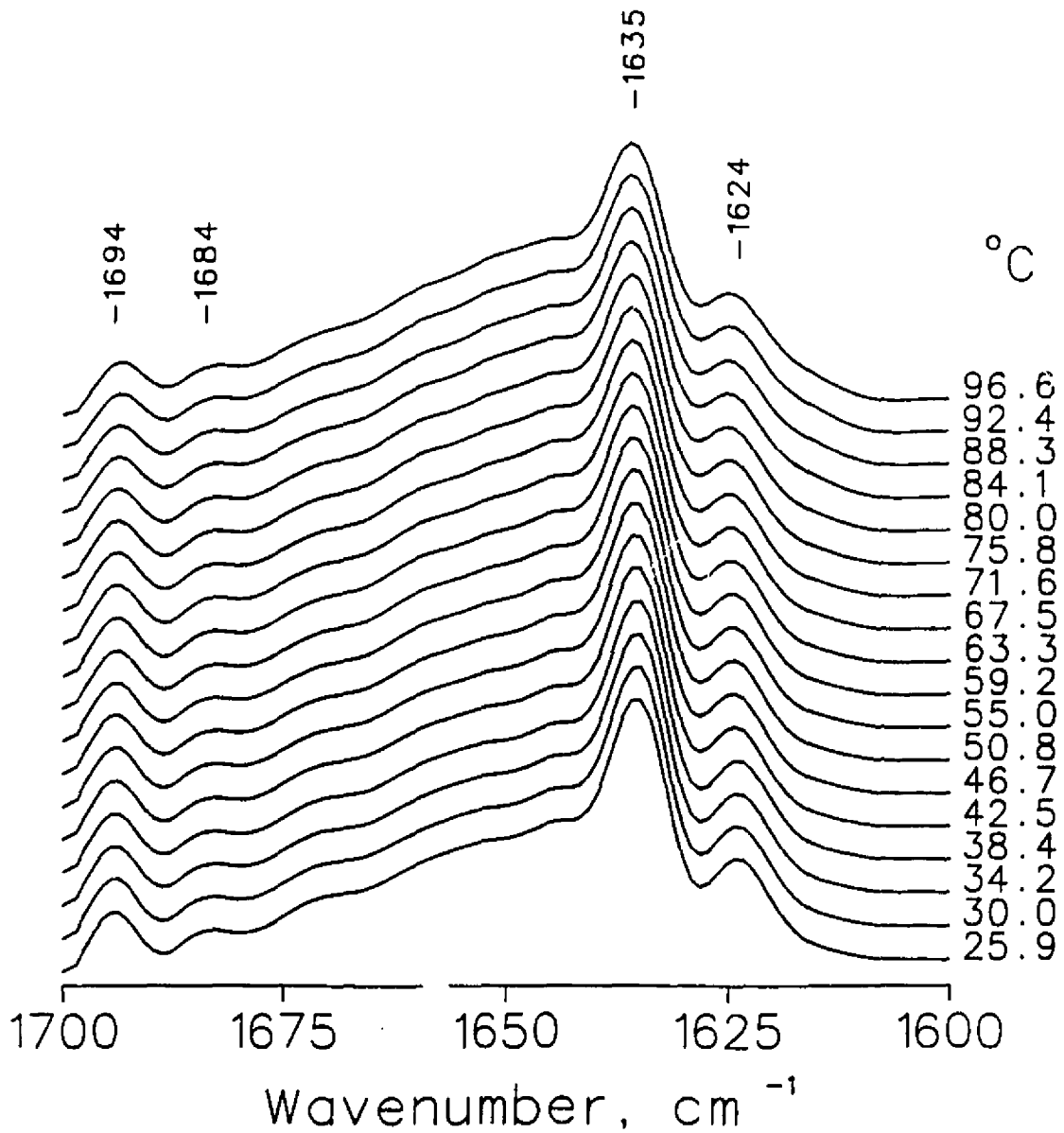

Figure 28 Amide I' Band in the FTIR Spectrum of Concanavalin A Heated in the Presence of 10% Fructose

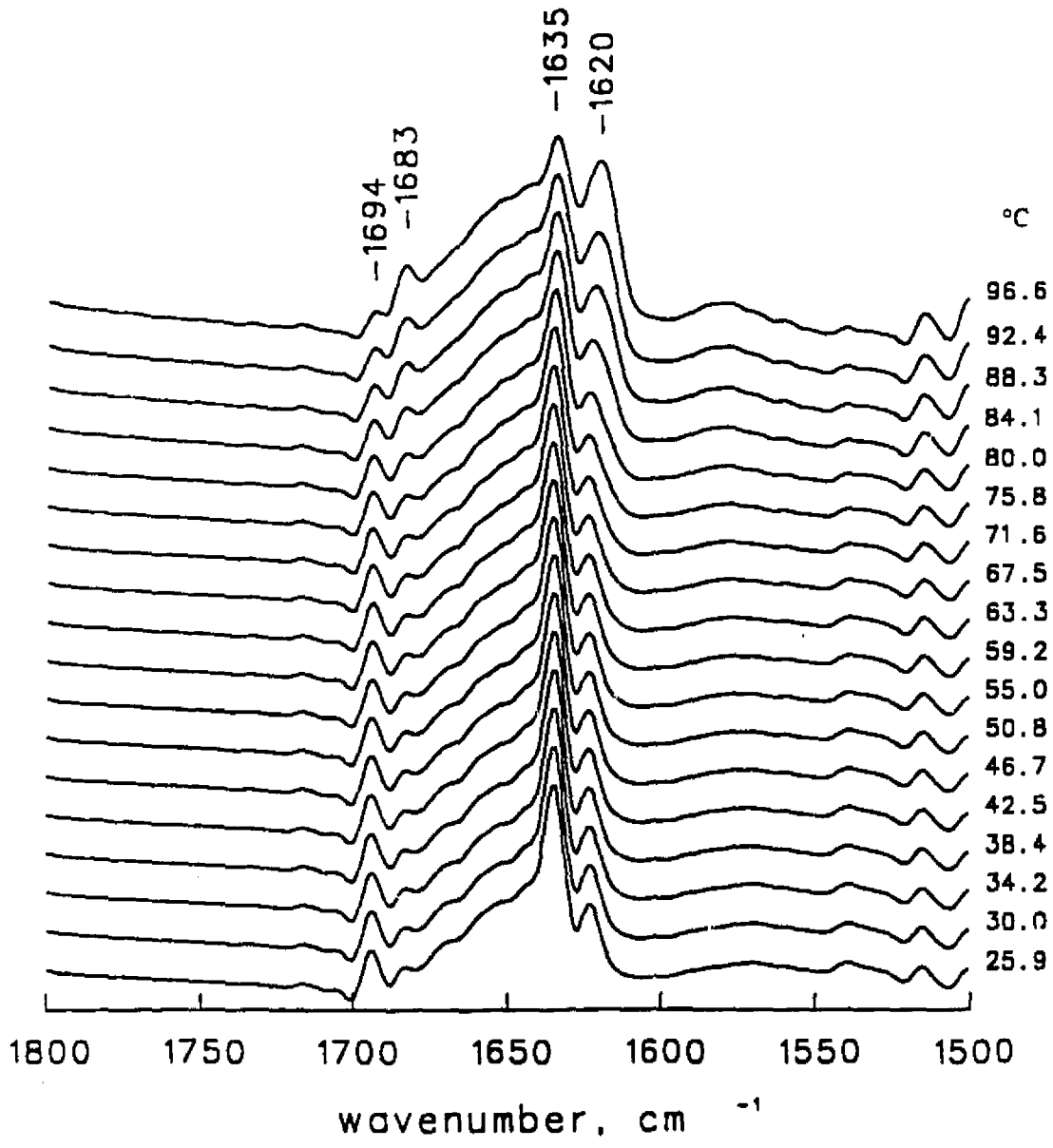

Figure 29 Amide I' Band in the FTIR Spectrum of Concanavalin A Heated in the Presence of 10% Sucrose

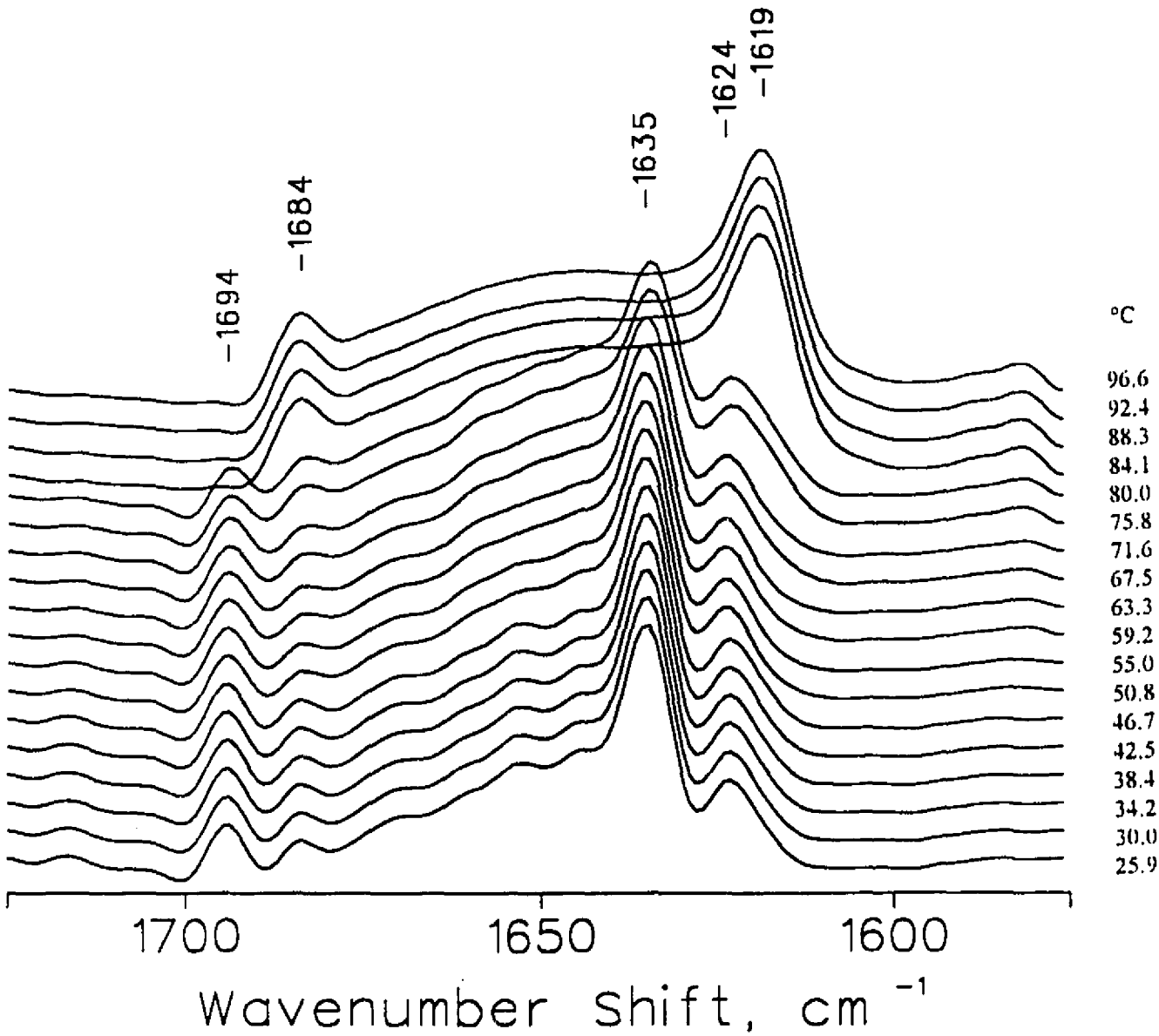

Figure 30 Amide I' Band in the FTIR Spectrum of Concanavalin A Heated in the Presence of 10% Galactose

Figure 31, shows the spectra of Con A in the presence of mannose, fructose, sucrose and galactose at 25 ^{o}C. It can be seen from this figure that these sugars have no apparent effect on the secondary structure of Con A at 25 ^{o}C. However, with the exception of galactose, these sugars confer some stability against the thermal denaturation of Con A, and both fructose and mannose appear to inhibit unfolding of Con A even at temperatures that normally result in complete denaturation of the protein. It may be postulated that the stabilizing effect of mannose is due to its specific binding to Con A. However, fructose, which has a much lower binding affinity to Con A than mannose, shows a much greater stabilizing effect. Thus, although the stabilizing effect appears to be sugar dependent, it does not correlate to the sugar binding affinity of Con A. Thus, the stabilizing effect may be attributed in part or solely to the hydrogen bonding propensity of the sugar binding to the protein. Alternatively, the sugar may bind intermolecularly with itself and with its surrounding water molecules, creating a network that stabilizes the structure of the protein. The presence of the open form of sugars at high temperatures may also play a key role in stabilizing the secondary structure (Table 7). In comparison with the other sugars investigated fructose has the highest concentration of open-chain form (Yaylayan & Ismail 1992), and as discussed above the increase in the open chain form with temperature is observed in Fig. 28. This suggests the possibility that the remarkable thermal stability of Con A in the presence of fructose may result from the presence of the open-chain form. Further experiments would be required to elucidate the mechanism by which fructose confers stabilizing its effect and to investigate the possible role of the open-chain form.

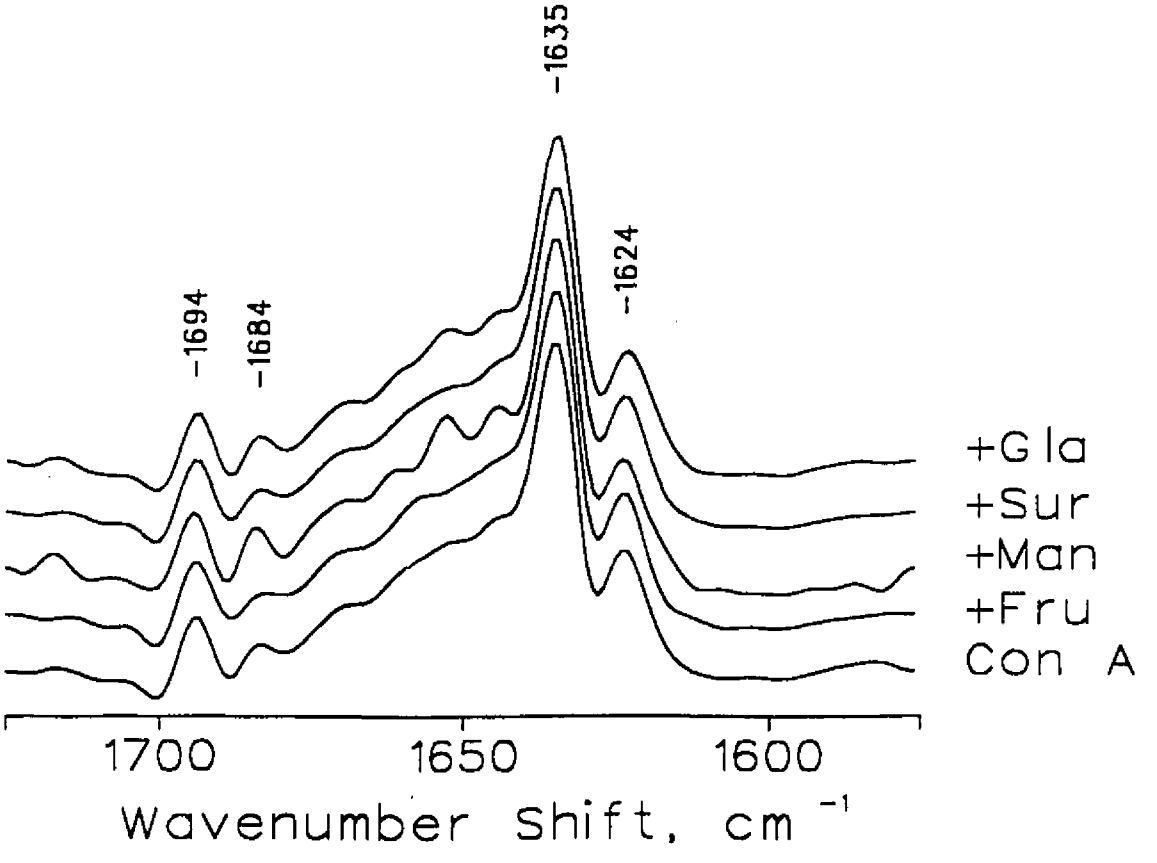

Figure 31 Spectra of Concanavalin A with Several Sugars at 25 °C

TABLE 7. The Thermal Stability of Con A as a Function of Sugars as Monitored by Changes in the Amide I' Band Profile

Con A + Sugar	1618 cm^{-1} (O) °C	1624 cm^{-1} (L) °C	1635 cm^{-1} (L) °C	1684 cm^{-1} (O) °C	1695 cm^{-1} (L) °C
Con A	80.0	80.0	84.1	⇑	84.1
Mannose	84.1	⇓	-	⇑	⇓
Fructose	-	⇐	-	⇑	-
Sucrose	84.1	88.3	-	⇑	⇓
Galactose	80.0	80.0	84.1	⇑	84.1

(O) Temperature at which band appears. (L) Temperature at which band completely disappears.

(⇑) Increase in band intensity with increasing temperature.

(⇓) Decrease in band intensity with increasing temperature. (⇐) Shift in the band position to lower wavenumber.

CHAPTER 5 CONCLUSIONS

Fourier transform infrared spectroscopy gives extensive information on protein secondary structure when coupled with Fourier self-deconvolution method for spectral analysis.

The secondary structure of Con A has been shown to be affected by factors such as temperature, pH, ionic strength, pressure and the presence of sugars. This work monitored the effects of the above factors on the thermal stability and secondary structure of Con A by differential scanning calorimetry (DSC) and FTIR spectroscopy. DSC studies indicate that the thermal stability of Con A is enhanced in the presence of salts, sugars and by pH. Changes in the secondary structure of Con A as a function of concentration, sugars, ionic strength and temperature were also investigated by Fourier Transform Infrared Spectroscopy. The conformation-sensitive amide I band, usually observed between 1600-1700 cm^{-1}, is the most important region for studies of the secondary structure of proteins. It was found that concentration, sugars and metal ions had no effect on the structural conformation of concanavalin A at room temperature. Variable-temperature FTIR studies of the thermal characteristics of con A as function of pH and sugars, showed that maximum thermal stability is achieved under alkaline conditions (pH 8-10). It was also found that the sugars confered remarkable thermal stability to Con A and the stabilizing effect of the sugar was not dependent on the sugar binding specificity of the lectin.

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