STUDIES OF THE REFOLDING OF CYTOCHROME C

by

Jorge Babul

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate College of The University of Iowa

May, 1971

Thesis supervisor: Associate Professor Earle Stellwagen

ABSTRACT

The folding of polypeptides from the unfolded to the native conformation was studied using cytochrome c as a model. Acidification of cytochrome c to pH 2.0 at low ionic strength results in an unfolded conformation. At this pH the protein has a reduced viscosity of 22 ml/g, characteristic of an extended chain, and a Soret maximum of 395 nm, characteristic of a high spin complex. Upon increasing the pH from pH 2.0, a sharp decrease in the reduced viscosity is observed reaching the value characteristic of the native molecule by pH 3.5. This structural change is accompanied by an increase in the Soret maximum to the value characteristic of the native protein, 410 nm.

The kinetics of folding of the unfolded protein were studied using a stopped-flow spectrophotometric technique. Absorbance changes accompanyihg the folding of the polypeptide caused by sudden changes in pH from pH 2.0 to pH 3-7, were followed. The refolding of the protein occurs very rapidly; the absorbance changes leading to the native conformation occur with a half time of about 3 sec. Analysis of the kinetic curves shows the presence of stable intermediates between unfolded and native molecules.

Comparison of the absorbance changes associated with the formation of each intermediate with the absorbance changes that accompany the formation of heme complexes gives an indication of the chemical nature of the intermediates and suggests a mechanism of folding. The results are consistent with a mechanism $U \xrightarrow{k_1} X_1 \xrightarrow{k_2} X_2 \xrightarrow{k_3} N$ where U and N represent unfolded and native molecules, respectively, and X_1 and X_2 are the detectable intermediates. X_1 is proposed to be a complex where one of the native ligands, HIS 18, is coordinated to the heme iron. X_2 is proposed to represent a substantially folded state where both native ligands, HIS 18 and MET 80, are coordinated to the heme iron. The value of the rate constants k_1, k_2, and k_3, for the refolding process at pH 7 and 25°, are in a ratio of approximately $k_1 : k_2 : k_3 =$ 1,000 : 300 : 1. The rate of the refolding reactions is found to increase upon increasing the final pH of the reaction mixture in the pH range 3-7.

Abstract approved: Earle Stellwagen, Thesis supervisor

Assoc. Prof. of Biochem., title and department

Feb. 11, 1971 date

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Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Jorge Babul

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy in the Department of Biochemistry at the May 1971 graduation.

Thesis committee: Earle Stadtman
Thesis supervisor

H. C. Hagenkamp
Member

George Kalnitsky
Member

Alex Montgomery
Member

H. B. Bull
Member

Frederick Dahl
Member

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LIST OF ABBREVIATIONS

CM MET: carboxymethyl methionyl

CM_2 HIS: dicarboxymethyl histidyl

cytochrome *c*: horse heart ferricytochrome *c*

GuHCl: guanidine hydrochloride

ORD: optical rotatory dispersion

INTRODUCTION

Proteins are natural high molecular weight polymers made up of twenty common amino acids linked by peptide bonds. When proteins are dissolved in aqueous solvent at neutral pH, most of them are found to be compact particles almost spherical in shape (1) which are characterized as "native" conformation. Among the interactions contributing to the stabilization of the native conformation are hydrogen bonds, ionic interactions, and hydrophobic interactions. Some proteins, in addition, are stabilized by intrachain covalent bonds such as disulfide bonds or coordinate covalent bonds. Theoretical calculations (1-3) show that the total free energy change involved in the transition from a random conformation having no intrachain bonds to a folded native conformation is surprisingly small, ranging from 10 to 20 kcal/mole for small proteins. The presence of one or more crosslinks would reduce the conformational entropy of the unfolded molecule (4), thus increasing the stability of the native structure. Of the non-covalent interactions, hydrophobic interactions make the largest contribution to the stabilization of the native conformation and can by themselves account for the stability of the globular conformation (1-3). A

thermodynamically more stable situation arises when the hydrophobic residues are clustered in the interior of the protein molecule rather than in the aqueous surroundings. This is found to be the case for several proteins upon examination of their structures at near-atomic resolution (5).

In general, proteins can be discussed in terms of at least three levels of organization. The covalent structure of a protein, i.e. the sequence of amino acid residues forming the backbone of the polypeptide, is referred to as the primary structure. The tendency of the backbone to locally fold into regular conformations such as an α-helix or a pleated sheet is termed secondary structure. The way in which the entire polypeptide chain is folded into a compact three dimensional shape is defined as the tertiary structure. Although there are no clear limits between these different levels of structure which may not be present in every protein, this division is useful for discussing the structure of proteins and for describing the events accompanying their unfolding or refolding.

Since the ΔG^{o} of folding or unfolding is small, proteins which show reversible transformation will probably undergo appreciable variation of structure in solution. If this is the case they cannot be said to have a unique native conformation and there will exist a family

of conformations having similar free energies (6). Proteins are known to have a flexible structure, their side chains are somewhat free to rotate, specially those in contact with the solvent. The peptide backbone must also experience local fluctuations in conformation, with temporary loosening of the structure in different regions of the molecule. Changes in conformation of this nature are necessary to explain the different hydrogen exchange rates observed with several proteins (7). Also, the binding properties of proteins like serum albumin suggest (8) the presence of a large number of conformations having nearly equivalent free energies. In addition, an equilibrium between at least two conformations is used to explain the allosteric behavior of some enzymes (9) also suggesting that there is not a unique conformation in solution.

In relation to the extent and rate of these fluctuations in structure, hydrogen exchange studies (6) indicate that fluctuations of a protein between the native conformation and a completely unfolded form does not occur to an appreciable extent over long periods of time. When a rapid equilibrium exists between two conformations, these structures will not be detected by ordinary techniques and only an average state is measured.

As discussed above, proteins in their native state are found to have a high degree of order resulting from a large number of interactions. This raises the question of

what directs the folding of polypeptide chains. At the present time the problem is understood only in broad terms. Information on the origin of the secondary and tertiary structure of proteins has been obtained experimentally by converting unfolded polypeptide chains into their native and biologically active conformations. Successful experiments of this type performed with several proteins (10) support the hypothesis that the three dimensional conformation is determined solely by the primary sequence and that the native conformation of proteins corresponds to the thermodynamically most stable conformation. Thus, under appropriate conditions, the transition from an unfolded polypeptide to a folded structure is shown to be a spontaneous process, driven by the difference in free energy of conformation between the unfolded and folded forms. Whether these conclusions derived from reversible denaturation experiments apply to all proteins or to only some proteins has not been ascertained. In some cases other factors, such as a cofactor or a prosthetic group, may be necessary for the formation of the native conformation.

Although it seems that the three dimensional structure is mainly determined by the amino acid sequence, the guiding principles of the correspondence between sequence and folding have not yet been discovered. As suggested by Phillips (11), it is possible that the three dimensional

structure is formed in some sequential fashion as the polypeptide grows on the ribosome. Part of the chain near the amino end could "freeze" into a form that would remain unchanged during the process of biosynthesis, and it would serve as a nucleus for folding of the rest of the chain. If this is the case, the structure of fragments of proteins including the amino terminus should show a structure similar to the one that that segment has in the native conformation. Studies of this kind (12-15) suggest that proteins do not start to assume their native conformations during biosynthesis, as suggested by Phillips, until the polypeptide is completely biosynthesized or nearly so.

One of the important consequences of the reversibility of protein denaturation discussed above, is that the folding process may be followed *in vitro* and thus provides an experimental tool for studying the kinetics and mechanism of the refolding process. Proteins exhibiting reversible unfolding can be used as models for the folding of newly-biosynthesized polypeptide chains and the times required for this transition can be compared with the process *in vivo*. Although no direct measurements of *in vivo* rates of folding are available, the time required for the total biosynthesis of proteins has been demonstrated to be, of the order of seconds to minutes (16,17). Only a few kinetic measurements of folding of proteins from an unfolded state to their native conformation have been

reported. Most estimates of refolding rates have been made indirectly by combining equilibrium and kinetic measurements with the assumption that the transition is a two-state process (18,19). Direct kinetic measurements of a few proteins (20-23) show that folding can be a relatively fast process, and that intermediate conformations exist.

In this study, equilibrium and kinetic measurements of the refolding of cytochrome c were performed in an effort to develop a system which could be used to study the kinetics and mechanism of refolding. Several features of cytochrome c makes this protein a suitable model for such studies.

1) It is a small protein composed of 104 amino acid residues.

2) Its native conformation is well characterized. Recent X-ray analysis of the protein (24) at 2.7 A^{o} resolution show the path of the polypeptide chain and the location of all side chains. The X-ray study also demonstrates that cytochrome c has the conformation of a typical globular protein in which the hydrophobic side chains are concentrated in the interior of the molecule and ionic and polar side chains constitute the surface. The correlation of the results of equilibrium and kinetic studies of the refolding reaction with the structural information of this model will help to elucidate the

mechanism of the folding process.

3) The protein does not possess disulfide bonds, whose formation is known to be slow (10).

4) Physical and chemical measurements suggest that the protein can be reversibly unfolded (25a).

5) Unlike several other hemoproteins, cytochrome c has the heme group covalently attached to the polypeptide backbone via two thioether bridges formed between CYS residues 14 and 17 and the heme vinyl side chains in positions 2 and 4. As shown by the X-ray study, the heme is the center of the molecule with the polypeptide chain wrapped around it in two halves: residues 1-47 to the right and 48-91 to the left of the heme. The heme is located in the resultant crevice with one edge exposed to the surroundings. The rest of the chain, residues 92-104, forms the top of the molecule. Surrounding the heme on all sides, except the exposed edge, are closely packed hydrophobic residues. Four of the heme ligands are provided by the porphyrin's nitrogens and the fifth and sixth ligand are provided by a nitrogen of HIS 18 and the sulfur atom of MET 80, respectively. Changes in the absorbance of the heme moiety accompanying conformational changes allow the heme to be used as a natural reporter group.

6) The availability of a great variety of natural sequenced variants of cytochrome c provides a series of

homologs to evaluate the role of particular amino acid residues in the folding mechanism.

This work describes equilibrium and kinetic experiments relating absorbance changes with changes in the structure of cytochrome c over the pH range 2-9. Model hemopeptides and chemically modified cytochrome c were also investigated in order to elucidate the nature of the refolding process.

RESULTS

Equilibrium Studies

A. Unmodified Protein

1. Viscosity

The effect of pH on the reduced viscosity of cytochrome c in the absence of added salt is shown in Figure 1. When the pH was decreased from neutrality the viscosity remained at a constant value of 4.5 ml/g until pH 3. Below this pH value a sharp increase was observed reaching a maximum value of 23.5 ml/g at pH 2.2. This value is consistent with an extensive unfolding of the molecule and it is higher than a value of 15.1 ml/g calculated for a random coil of 104 amino acid residues (25b). The difference between the experimental and calculated values is probably due to the rod-like shape that the molecule acquires at low pH. In cytochrome c, 24 of the 104 amino acid residues can carry a positive charge at low pH, so a considerable expansion of the molecule should be expected in this condition as a result of electrostatic repulsion. Further lowering of the pH from pH 2.2 (see figure 1), causes a decrease in viscosity. This effect is due to the partial shielding of the charge

Figure 1. Reduced viscosity of cytochrome c, η_{sp}/c, as a function of pH. Decreasing pH in H_2O (O), increasing pH at ionic strength 0.007 (●), and in 0.3 M KCl (□).

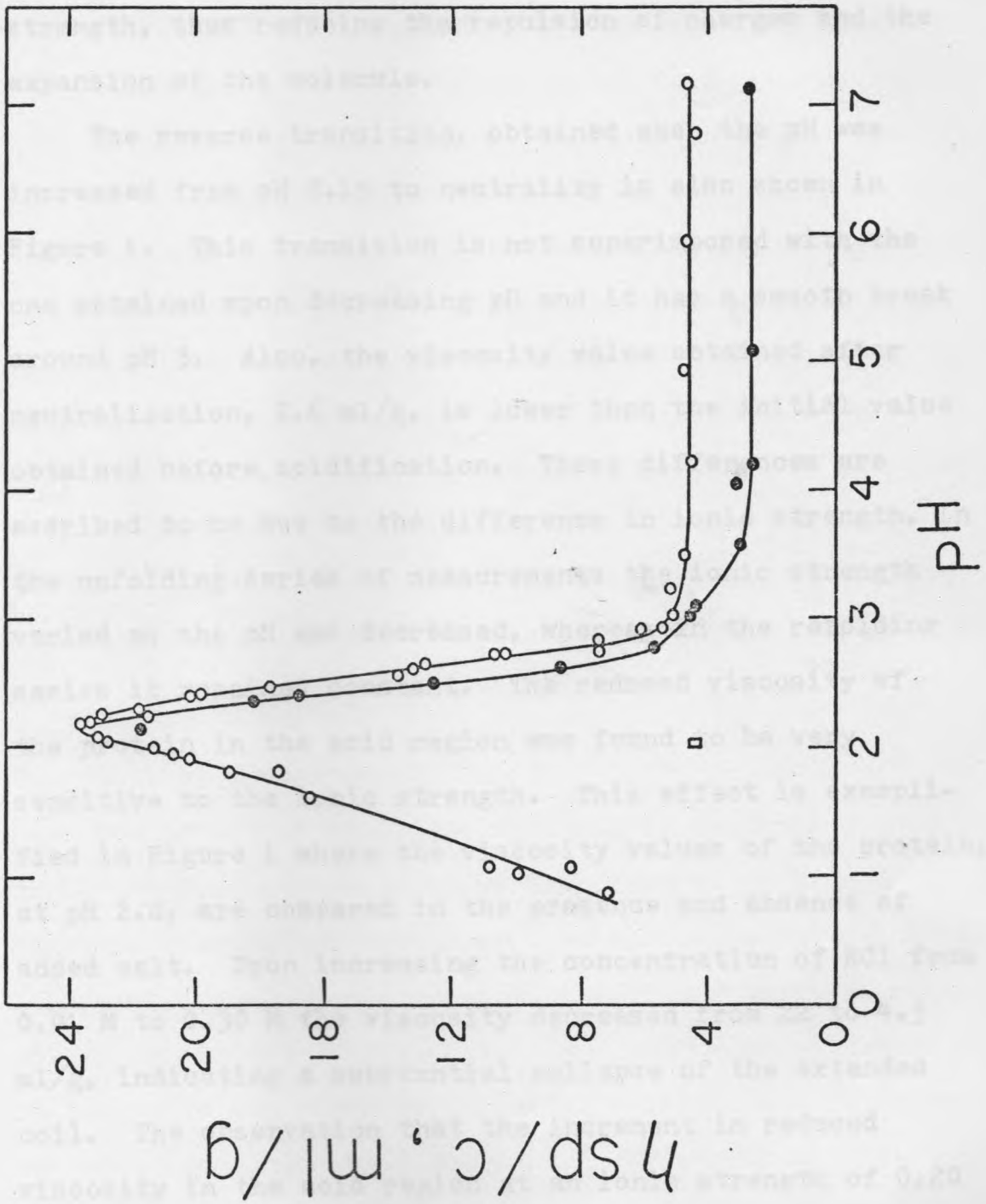

Figure 1

repulsion by negative ions (26). Lowering the pH from pH 2.2 would only have the effect of increasing the ionic strength, thus reducing the repulsion of charges and the expansion of the molecule.

The reverse transition, obtained when the pH was increased from pH 2.15 to neutrality is also shown in Figure 1. This transition is not superimposed with the one obtained upon decreasing pH and it has a smooth break around pH 3. Also, the viscosity value obtained after neutralization, 2.6 ml/g, is lower than the initial value obtained before acidification. These differences are ascribed to be due to the difference in ionic strength. In the unfolding series of measurements the ionic strength varied as the pH was decreased, whereas in the refolding series it remained constant. The reduced viscosity of the protein in the acid region was found to be very sensitive to the ionic strength. This effect is exemplified in Figure 1 where the viscosity values of the protein, at pH 2.0, are compared in the presence and absence of added salt. Upon increasing the concentration of KCl from 0.01 M to 0.30 M the viscosity decreased from 22 to 4.5 ml/g, indicating a substantial collapse of the extended coil. The observation that the increment in reduced viscosity in the acid region at an ionic strength of 0.20 is not consistent with an extensive unfolding of the molecule (27) is in agreement with the above results. At

neutral pH the native molecule showed a reduced viscosity of 2.6 ml/g at an ionic strength of 0.03-0.27. The same viscosity value was obtained in the pH range 4-7 upon neutralization of the molecule from pH 2.15, suggesting that cytochrome c is reversibly unfolded in acid. The ability of the polypeptide to return to its native state after prolonged exposure to acid was investigated by maintaining the protein in an HCl solution of pH 2.0 for 14 hours (see Methods), and then measuring the reduced viscosity after neutralization. A value of 2.6 ml/g was obtained, demonstrating the stability toward acid and the reversibility of the transition. The elution behavior of the refolded protein on a Sephadex G-50 column after standing 8 hours at pH 2.0 corresponded to that of the native cytochrome c.

2. Spectral Transitions

The effect of pH on the spectrum of cytochrome c in the 250-750 nm region is shown in Figure 2. Profound spectral changes were observed upon unfolding and refolding of the protein. In the unfolded acidified state the heme moiety is exposed to the solvent and the protein ligands in the fifth and sixth coordination position are replaced by the solvent. These chemical and environmental changes about the heme group that occur upon acidification and neutralization are reflected in the ultraviolet, near

Figure 2. Equilibrium absorption spectra of native and unfolded cytochrome c. Native in H_2O pH 7.0 (——), unfolded in 0.01 M HCl (----), and refolded, obtained after exposure to 0.01 M HCl and neutralization to pH 7.0 (——). Concentrations: A, 1.4×10^{-5} M; B, 0.40×10^{-5} M; and C, 6.6×10^{-5} M.

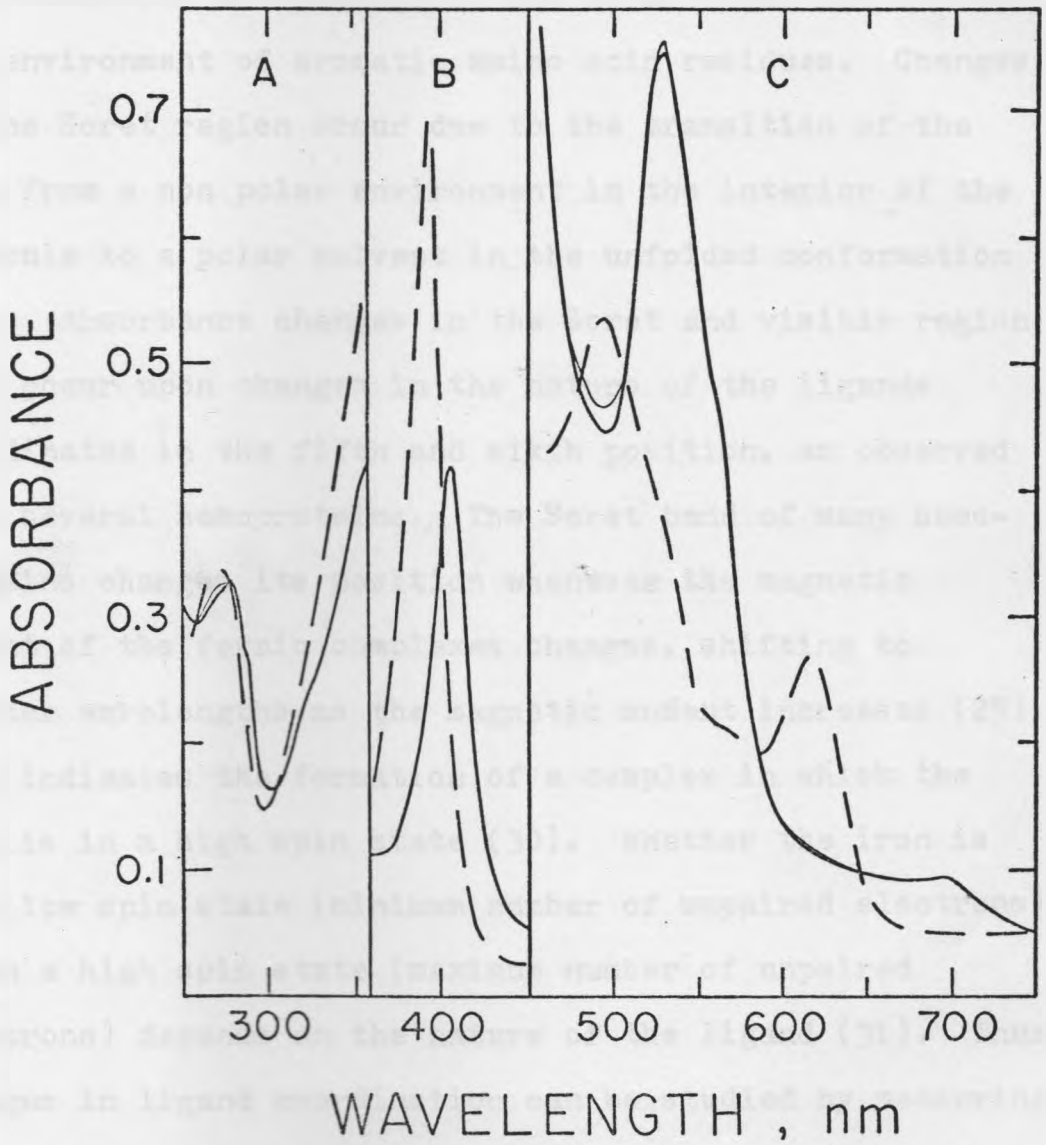

Figure 2

ultraviolet (Soret) and visible region of the spectrum of the molecule (Figure 2). The absorbance changes in the ultraviolet region are partly due to the change in environment of the heme group and also to the changes in the environment of aromatic amino acid residues. Changes in the Soret region occur due to the transition of the heme from a non polar environment in the interior of the molecule to a polar solvent in the unfolded conformation (28). Absorbance changes in the Soret and visible region also occur upon changes in the nature of the ligands coordinated in the fifth and sixth position, as observed with several hemoproteins. The Soret band of many hemoproteins changes its position whenever the magnetic moment of the ferric complexes changes, shifting to shorter wavelengths as the magnetic moment increases (29). This indicates the formation of a complex in which the iron is in a high spin state (30). Whether the iron is in a low spin state (minimum number of unpaired electrons) or in a high spin state (maximum number of unpaired electrons) depends on the nature of the ligand (31). Thus, changes in ligand coordination can be studied by measuring changes in the position of the Soret band. As seen in Figure 2, the Soret band shifts to a shorter wavelength upon acidification suggesting the above mentioned changes in ligand. As shown by magnetic measurements (32), the Soret shifts observed upon acidification of cytochrome c

are accompanied by changes in the spin state of the heme iron, the high spin complex appearing at low pH. In the visible region of the spectra of several hemoproteins a rise in intensity at 600-650 nm together with a fall in intensity at 550-600 nm is observed as the magnetic moment increases (33). The band at 600-650 nm appears when at least some of the ferric complex is in the high spin state and vanishes in the low spin complexes. Low spin complexes are formed by coordination of strong field ligands (34). Potential strong field ligands in cytochrome c include the imidazole group of histidine, the sulfur of methionine, the ε-amino group of lysine, and the indole of tryptophan (35). As seen in Figure 2, a band at 620 nm is formed upon acidification of the protein and disappears upon neutralization giving an indication of the spin state of the heme iron and of the nature of the coordination complex. Finally, a band of very low extinction at 695 nm is known to be conformationally sensitive and present only in the native protein (36). This band, as seen in Figure 2, disappears upon acidification.

In Figures 3 and 4 the spectra at intermediate stages between the unfolded and refolded states are shown in the Soret and visible regions of the spectrum. At pH 2, the Soret maximum is 394-395 nm, characteristic of a high spin complex in which the protein ligands, MET 80 and HIS 18, are replaced by the solvent, probably two chloride ions

Figure 3. Reverse titration of cytochrome c in the absence of extrinsic salt. pH: A - 2.16, B - 2.30, C - 2.37, D - 2.41, E - 2.46, F - 2.53, G - 2.65, H - 3.69, I - 7.10. Concentration: 5.5×10^{-6} M.

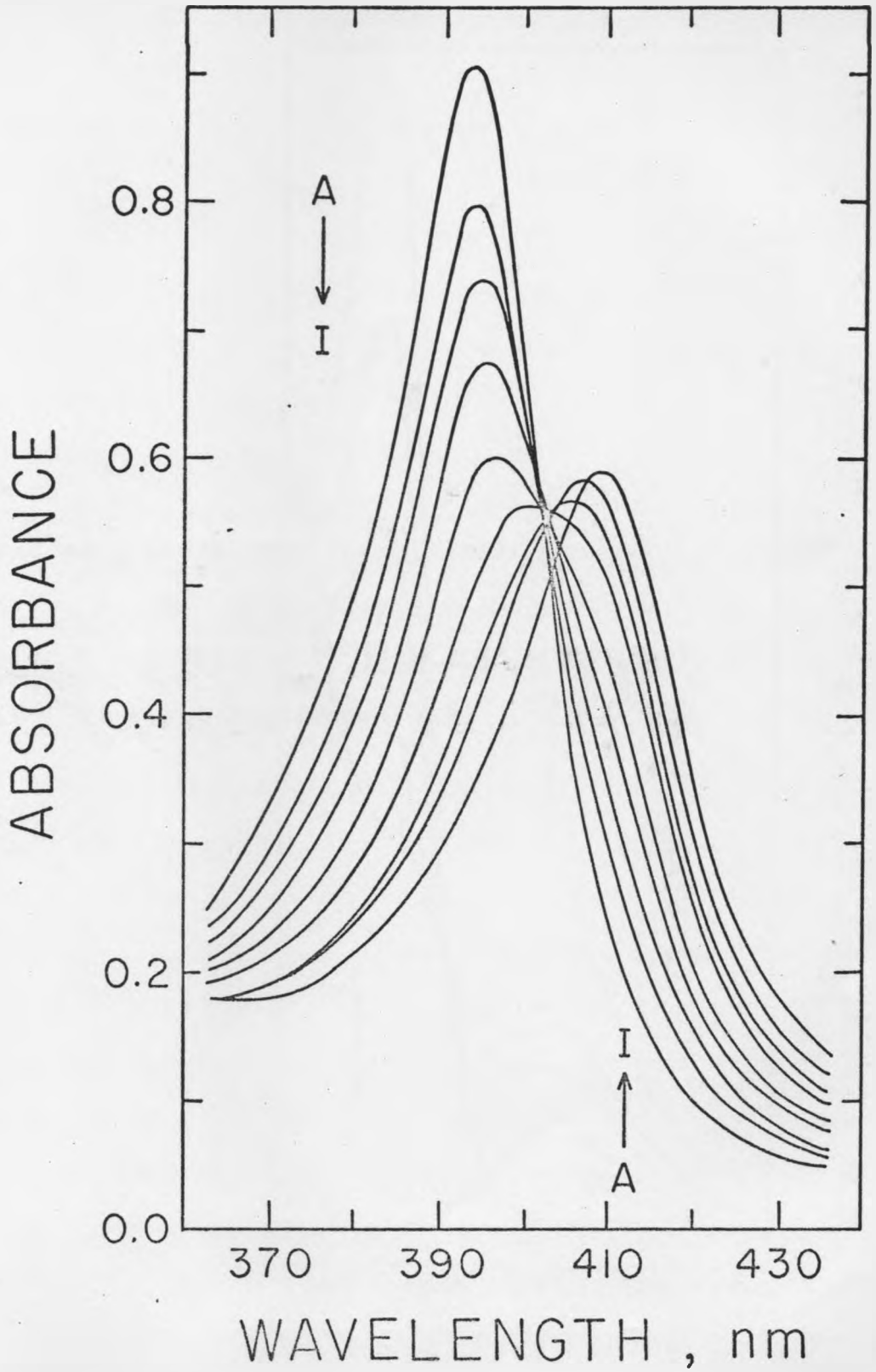

Figure 3

Figure 4. Reverse titration of cytochrome c in the absence of extrinsic salt. pH: A - 1.98, B - 2.35, C - 2.51, D - 2.70, E - 3.00, F - 7.05. Concentration: 2.9×10^{-4} M.

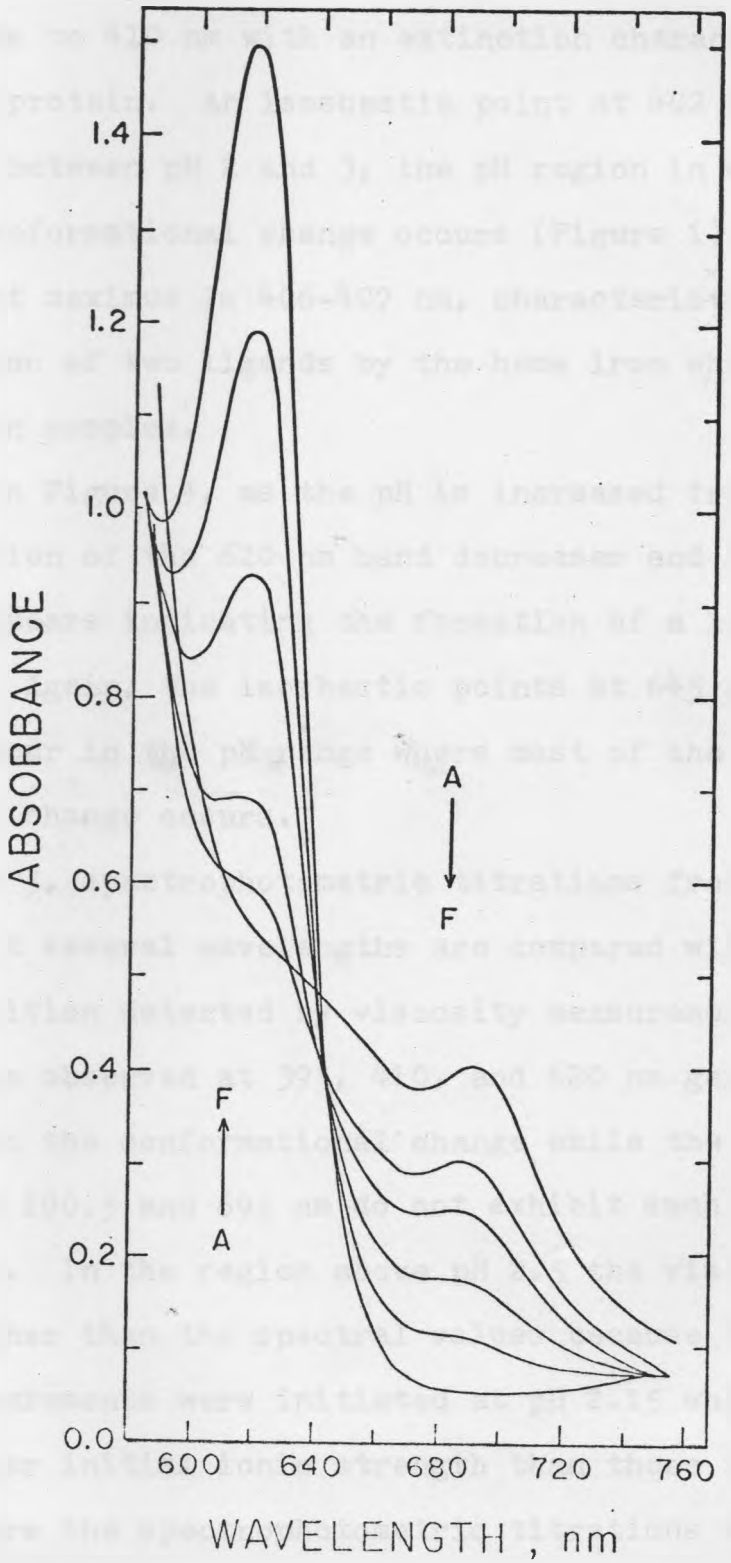
ABSORBANCE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
600
640
680
720
760
WAVELENGTH , nm
A
F
F
A

Figure 4

(32). Upon increasing the pH from pH 2 to 7, the Soret maximum returns to 410 nm with an extinction characteristic of the native protein. An isosbestic point at 402 nm is observed only between pH 2 and 3, the pH region in which most of the conformational change occurs (Figure 1). At pH 3, the Soret maximum is 406-407 nm, characteristic of the coordination of two ligands by the heme iron which form a low spin complex.

As seen in Figure 4, as the pH is increased from pH 2, the extinction of the 620 nm band decreases and the 695 nm band appears indicating the formation of a low spin complex. Again, the isosbestic points at 645 and 755 nm only occur in the pH range where most of the conformational change occurs.

In Figure 5, spectrophotometric titrations from about pH 2 to pH 7 at several wavelengths are compared with the spectral transition detected by viscosity measurements. The transitions observed at 395, 410, and 620 nm generally correspond with the conformational change while the transitions at 290.5 and 695 nm do not exhibit such a close correspondence. In the region above pH 2.5 the viscosity values are higher than the spectral values because the viscosity measurements were initiated at pH 2.15 which produces a lower initial ionic strength than those at pH 1.7-2.0 where the spectrophotometric titrations were started. Each of the reverse spectrophotometric titrations

Figure 5. Reverse spectrophotometric titrations at different wavelengths and reduced viscosity of cytochrome c. (●) η_{sp}/c, (○) 695 nm, (△) 620 nm, (■) 410 nm, (▲) 395 nm, (□) 290.5 nm. Ionic strength 0.007-0.020.

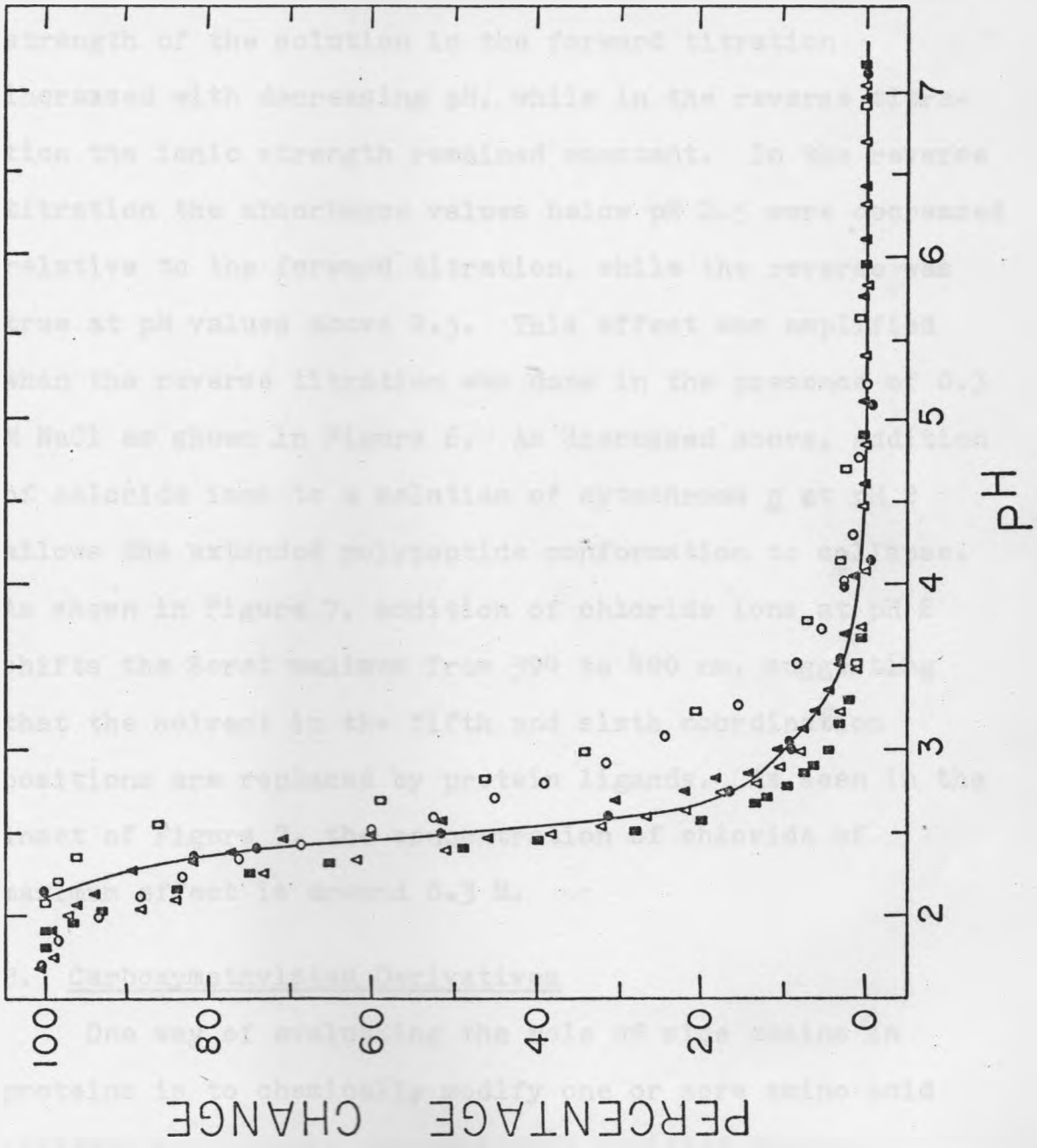

Figure 5

shown in Figure 5 is not superimposable with its forward titration, as shown in Figure 6 for the titrations at 395 nm. As noted in the viscosity experiments, the ionic strength of the solution in the forward titration increased with decreasing pH, while in the reverse titration the ionic strength remained constant. In the reverse titration the absorbance values below pH 2.5 were decreased relative to the forward titration, while the reverse was true at pH values above 2.5. This effect was amplified when the reverse titration was done in the presence of 0.3 M NaCl as shown in Figure 6. As discussed above, addition of chloride ions to a solution of cytochrome c at pH 2 allows the extended polypeptide conformation to collapse. As shown in Figure 7, addition of chloride ions at pH 2 shifts the Soret maximum from 394 to 400 nm, suggesting that the solvent in the fifth and sixth coordination positions are replaced by protein ligands. As seen in the inset of Figure 7, the concentration of chloride of maximum effect is around 0.3 M.

B. Carboxymethylated Derivatives

One way of evaluating the role of side chains in proteins is to chemically modify one or more amino acid residues and compare the variously modified products with the native protein. The effect of chemical modification of HIS 18 and MET 80, the ligands in the fifth and

Figure 6. Spectrophotometric titrations of cytochrome c at 395 nm in the presence and absence of extrinsic salt. Forward (●) and reverse (○) titrations with no extrinsic salt, reverse titration in the presence of 0.3 M NaCl (□).

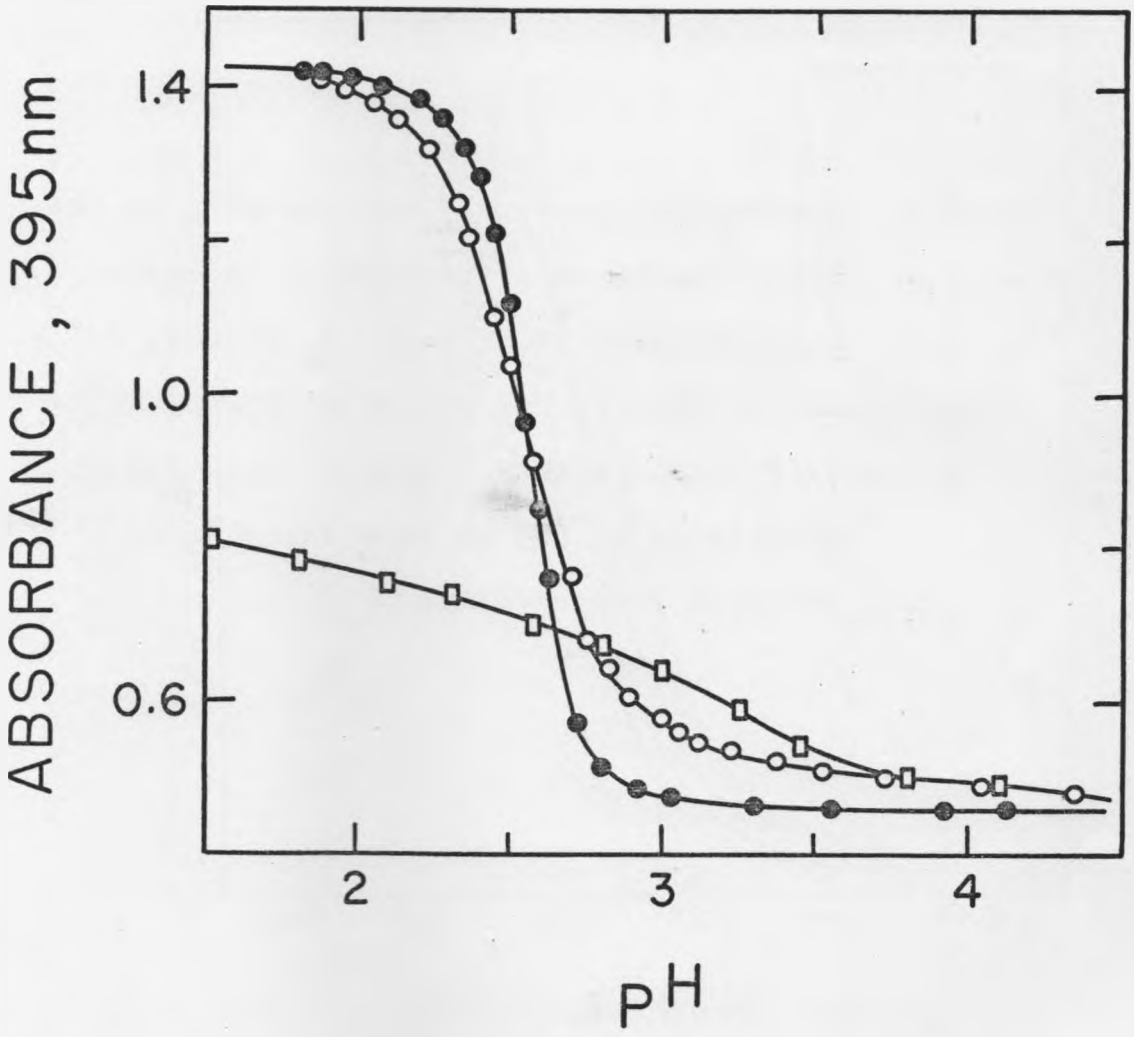

Figure 6

Figure 7. Absorption spectra of cytochrome c in the Soret region as a function of chloride concentration at pH 2.0. A, HCl; B, HCl + 0.03 M KCl; C, HCl + 0.05 M KCl; D, HCl + 0.10 M KCl; E, HCl + 0.30 M KCl. Inset, absorbance at 395 nm as a function of chloride concentration.

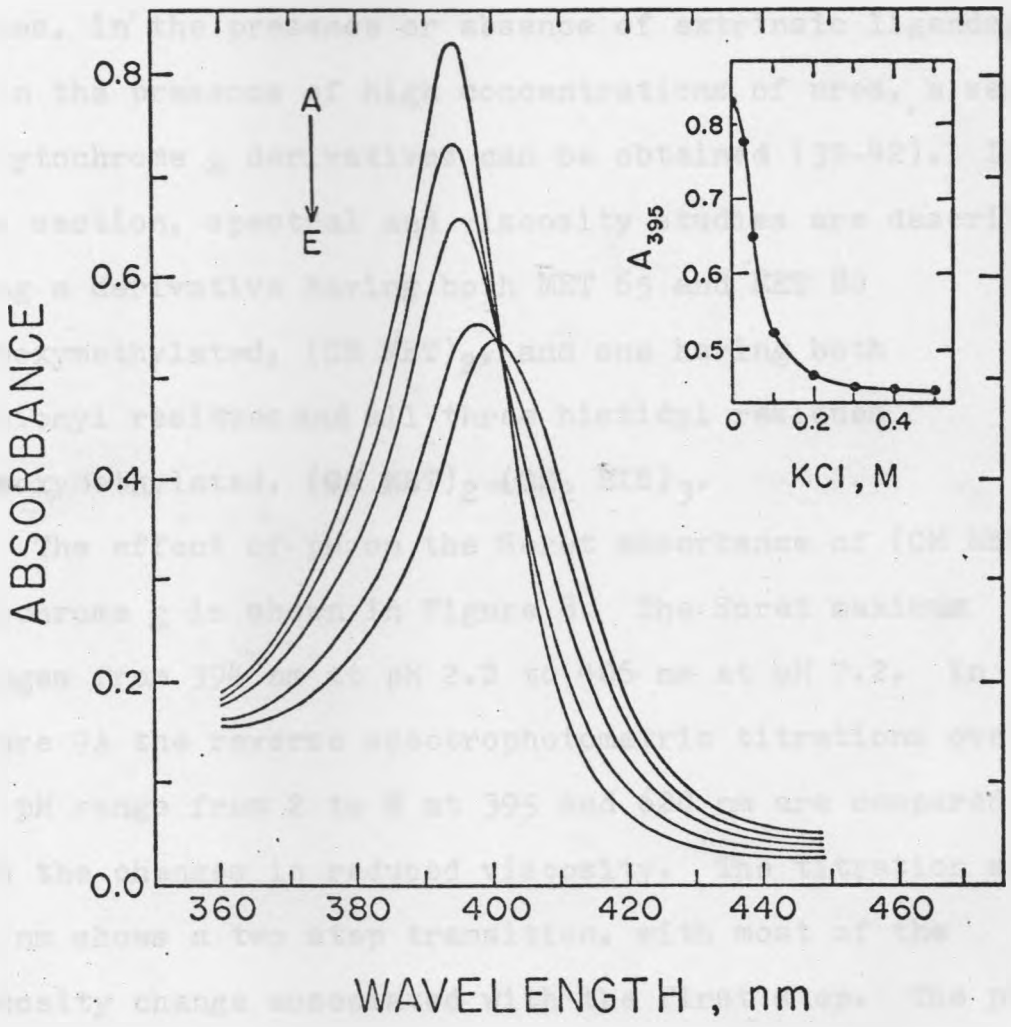
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
360
380
400
420
440
460
WAVELENGTH, nm
A
E
A_{395}
0.5
0.6
0.7
0.8
0
0.2
0.4
KCl, M

Figure 7

sixth position, was studied in order to evaluate the role of these residues in the structure of the protein. Modification by bromoacetate was chosen since the number and kinds of residues modified by this reagent can be easily controlled. By performing the reaction at different pH values, in the presence or absence of extrinsic ligands, or in the presence of high concentrations of urea, a series of cytochrome c derivatives can be obtained (37-42). In this section, spectral and viscosity studies are described using a derivative having both MET 65 and MET 80 carboxymethylated, $(CM\ MET)_2$, and one having both methionyl residues and all three histidyl residues carboxymethylated, $(CM\ MET)_2\ (CM_2\ HIS)_3$.

The effect of pH on the Soret absorbance of $(CM\ MET)_2$ cytochrome c is shown in Figure 8. The Soret maximum changes from 394 nm at pH 2.2 to 406 nm at pH 7.2. In Figure 9A the reverse spectrophotometric titrations over the pH range from 2 to 8 at 395 and 620 nm are compared with the changes in reduced viscosity. The titration at 395 nm shows a two step transition, with most of the viscosity change associated with the first step. The pK of the first spectral transition and of the viscosity change is the same, 2.9. Comparison of this value with the one obtained with the unmodified protein, 2.5, indicates that this carboxymethylated derivative is somewhat less resistant than the native protein to the unfolding

Figure 8. Reverse titration of $(CM\ MET)_2$ cytochrome *c* in the absence of extrinsic salt. A through E, $(CM\ MET)_2$ cytochrome *c*; F, native cytochrome *c* at pH 7.0. pH: A - 2.19, B - 2.61, C - 3.05, D - 4.69, E - 7.25. Concentration: 5.0×10^{-6} M.

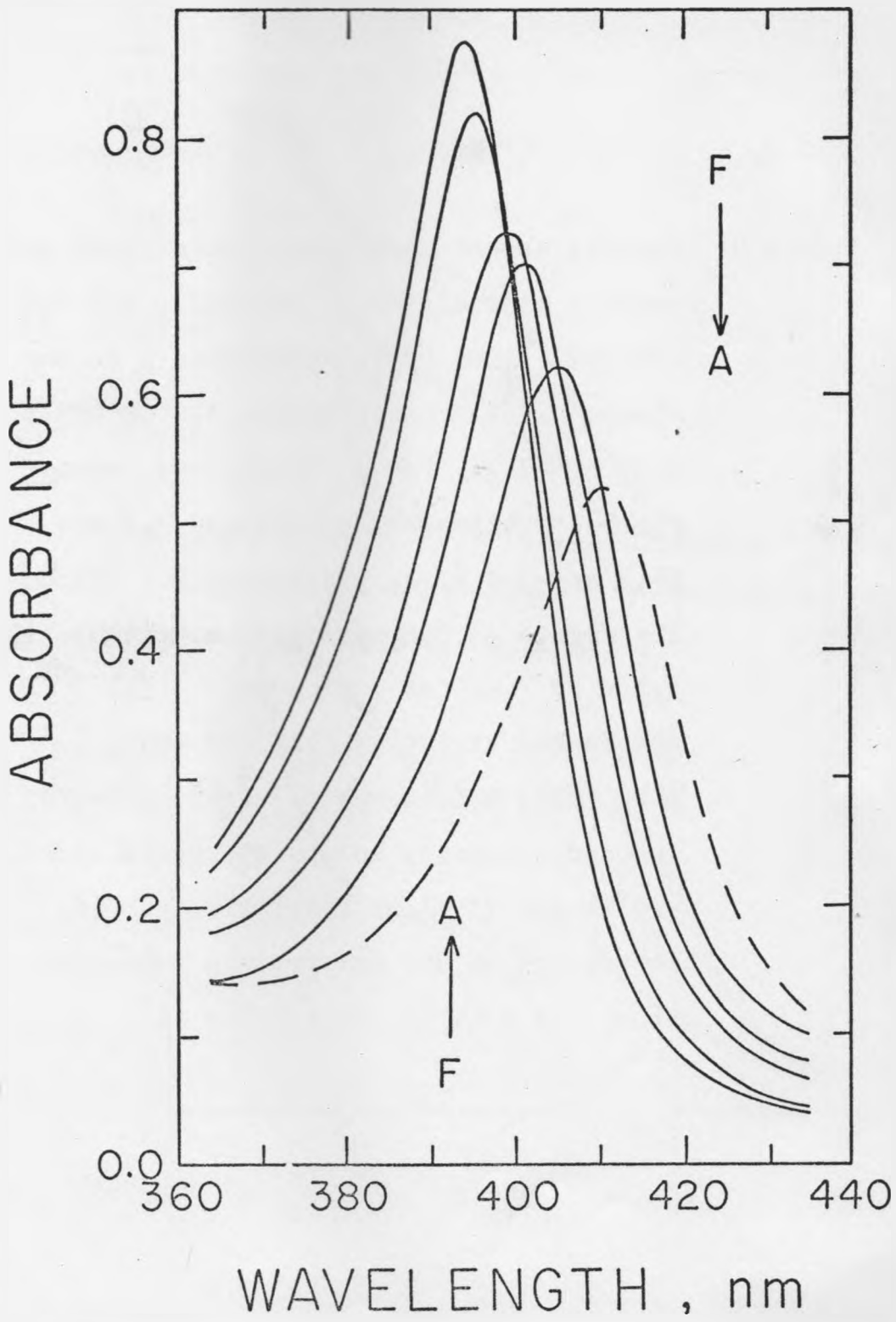
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
360
380
400
420
440
WAVELENGTH , nm
F
A
A
F

Figure 8

Figure 9. Reverse spectrophotometric titrations and reduced viscosities of $(CM\ MET)_2$ and $(CM\ MET)_2\ (CM_2\ HIS)_3$ cytochrome c in the absence of extrinsic salt. A, $(CM\ MET)_2$ cytochrome c. (●) $\eta sp/c$; the reduced viscosity values at pH 2.2 and 7.3 are 24.4 and 3.2 ml/g, respectively. (○) Absorbance at 395 nm; protein concentration, 5.7×10^{-6} M; $\Delta\varepsilon$, 10×10^4. (△) Absorbance at 620 nm. B, $(CM\ MET)_2\ (CM_2\ HIS)_3$ cytochrome c. (●) $\eta sp/c$, the reduced viscosity values at pH 2.0 and 8.3 are 24 and 15 ml/g, respectively. (○) Absorbance at 390 nm; protein concentration, 4.4×10^{-6}; $\Delta\varepsilon$, 1.3×10^5.

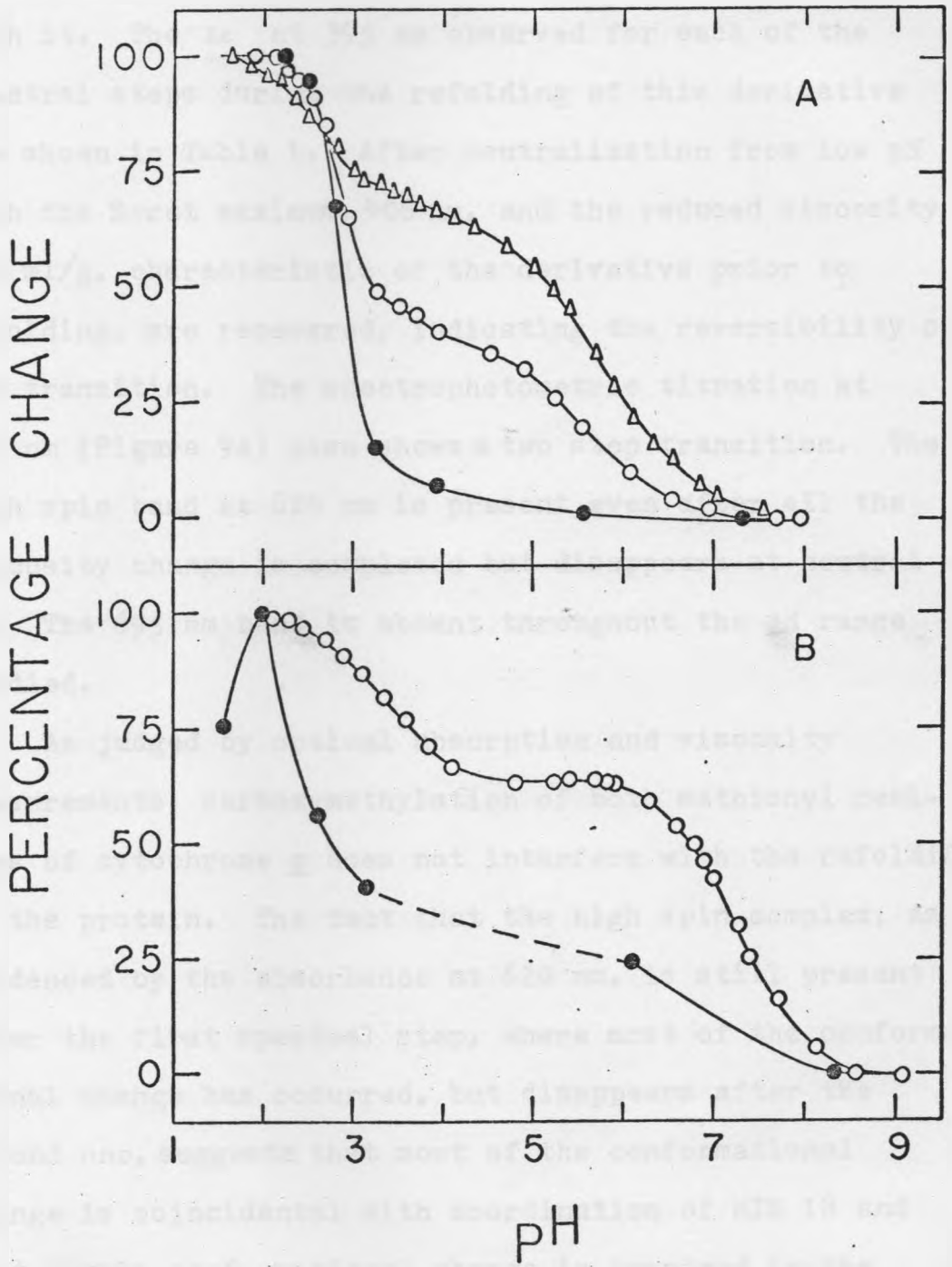

Figure 9

action of acid. The second spectral transition has a pK value of 5.6 and little viscosity change is associated with it. The $\Delta\varepsilon$ at 395 nm observed for each of the spectral steps during the refolding of this derivative are shown in Table 1. After neutralization from low pH both the Soret maximum, 406 nm, and the reduced viscosity, 3.2 ml/g, characteristic of the derivative prior to unfolding, are recovered, indicating the reversibility of the transition. The spectrophotometric titration at 620 nm (Figure 9A) also shows a two step transition. The high spin band at 620 nm is present even after all the viscosity change is completed but disappears at neutral pH. The 695 nm band is absent throughout the pH range studied.

As judged by optical absorption and viscosity measurements, carboxymethylation of both methionyl residues of cytochrome c does not interfere with the refolding of the protein. The fact that the high spin complex, as evidenced by the absorbance at 620 nm, is still present after the first spectral step, where most of the conformational change has occurred, but disappears after the second one, suggests that most of the conformational change is coincidental with coordination of HIS 18 and that little conformational change is involved in the coordination of the ligand in the sixth position. The ligand in this position can be the modified methionyl

residue or another strong-field ligand. It has been suggested (39) that the ionized carboxyl of the carboxymethyl group can largely neutralize the positive charge in the sulfur atom and allow the formation of a CM MET-heme iron coordinate covalent bond. Protonation of the carboxyl group would destabilize the coordination of the CM-MET ligand, a situation which may account for the second spectral transition having a pK of 5.6. Since this transition has a pK substantially above that of the major viscosity transition, it probably reflects a local conformational change. The similarity of the ORD spectra of the native protein and the $(CM\ MET)_2$ derivative (38) support this interpretation. Alternatively, the coordination of LYS 79 in $(CM\ MET)_2$ cytochrome c has been suggested based on examination of a Kendrew skeletal model of the protein (43). Although the pK of 5.6 obtained in this work is too low for a "normal" ε-amino group, such a lowered pK could be envisaged. Because the major part of a lysyl side chain is hydrophobic, this part will have a tendency to be buried in the interior of the protein structure, and if the hydrophobic part does not project outside sufficiently, the uncharged form of the group becomes stabilized relative to the charged form, and its pK will be altered. Also, the heme group, by combining only with the uncharged form of the residue, would lower the pK of the ionization.

The effect of pH on the Soret band of $(CM\ MET)_2$ $(CM_2\ HIS)_3$ cytochrome c is shown in Figure 10. The Soret maximum remained constant at 390 nm but the extinction decreased markedly with increasing pH. The spectra of this derivative at neutral pH is comparable with that observed for polymerized heme (44) and hemopeptides (45,46). In these studies, a decrease in the absorbance of the Soret band and the increase in absorbance of a broad band around 350 nm are found to be the characteristic features of heme polymerization. The similarity of spectral changes observed with the $(CM\ MET)_2$ $(CM_2\ HIS)_3$ derivative suggests that polymerization occurs upon neutralization. The spectrophotometric titration shown in Figure 9B suggests that this polymerization occurs in two steps with pK values of 3.3 and 7.2. This derivative had a high reduced viscosity; values of 24 and 15 ml/g were obtained at pH 2.0 and 8.3 respectively. Precipitation was observed in the pH range 3-6. This derivative remained a high spin complex throughout the pH range 2-9 as evidenced by the persistence of a band at about 620 nm. The extinction at 620 nm did not change significantly with pH.

C. Hemopeptides

Spectrophotometric titrations were performed with model hemopeptides in order to determine the absorption

Figure 10. Reverse titration of $(CM\ MET)_2$ $(CM_2\ HIS)_3$ cytochrome c in the absence of extrinsic salt. A-D, $(CM\ MET)_2$ $(CM_2\ HIS)_3$ cytochrome c; E, native cytochrome c at pH 7.0. pH: A - 1.99, B - 5.43, C 0 7.02, D - 9.07. Concentration: 5.7×10^{-6} M.

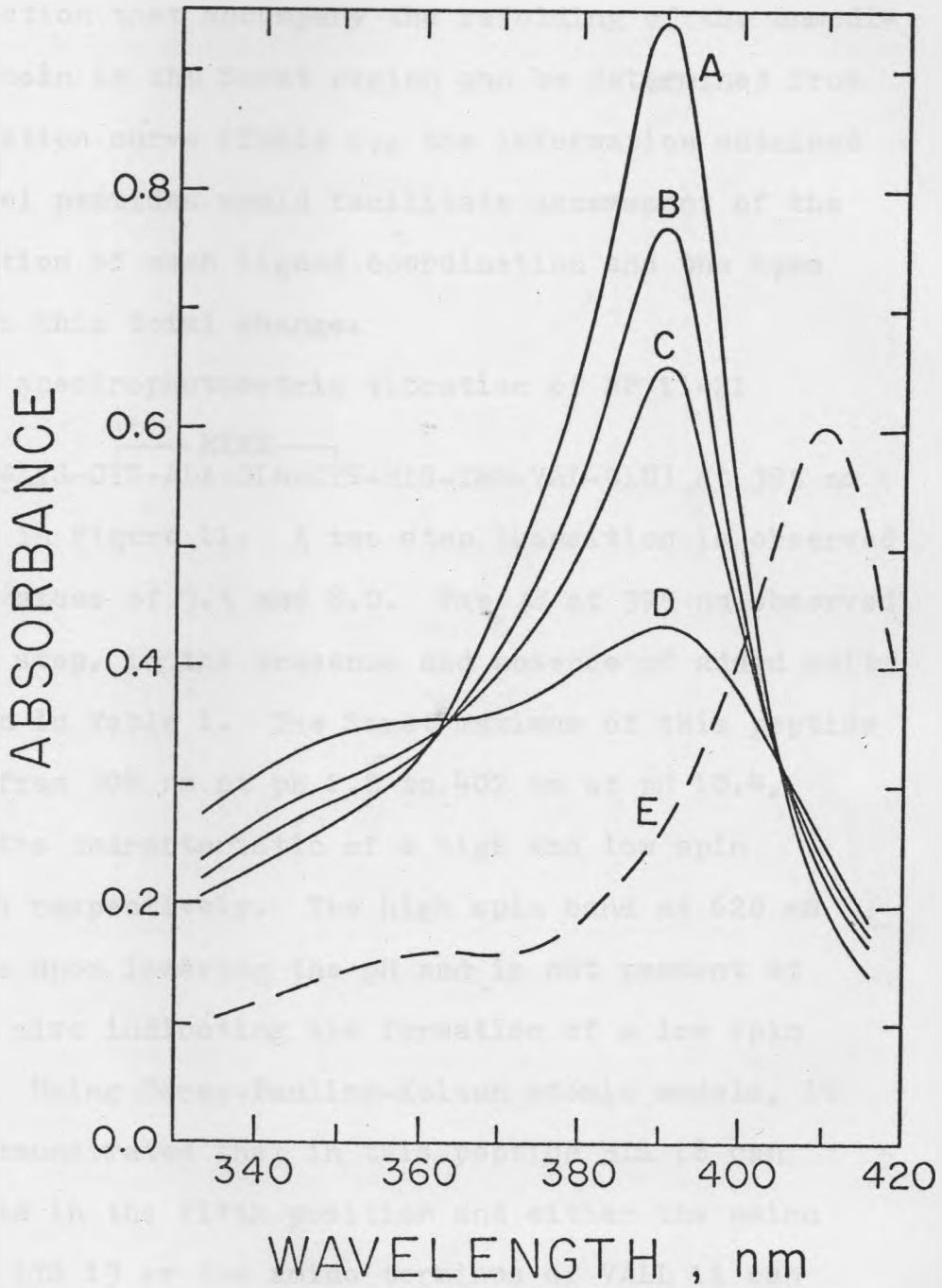
A
B
C
D
E
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
340
360
380
400
420
WAVELENGTH , nm

Figure 10

changes that accompany the coordination of ligands forming a low spin complex with the heme iron. Since the changes in extinction that accompany the refolding of the unmodified protein in the Soret region can be determined from its titration curve (Table 1), the information obtained with model peptides would facilitate accessment of the contribution of each ligand coordination and the heme burial to this total change.

The spectrophotometric titration of HP 11-21 (VAL-GLN-LYS-CYS-ALA-GLN-CYS-HIS-THR-VAL-GLU) at 395 nm is shown in Figure 11. A two step transition is observed with pK values of 3.5 and 8.0. The $\Delta\varepsilon$ at 395 nm observed for each step, in the presence and absence of added salt are shown in Table 1. The Soret maximum of this peptide changes from 394 nm at pH 1.8 to 407 nm at pH 10.4, wavelengths characteristic of a high and low spin complexes respectively. The high spin band at 620 nm decreases upon lowering the pH and is not present at pH 10.4, also indicating the formation of a low spin complex. Using Corey-Pauling-Koltun atomic models, it can be demonstrated that in this peptide HIS 18 can coordinate in the fifth position and either the amino group of LYS 13 or the amino terminus of VALL 11 can coordinate in the sixth position. The spectral pK values are in agreement with this proposal. The first step with

TABLE 1

Equilibrium $\Delta\varepsilon_{395}$ for Ligand Coordination to the Heme Iron[a]

Specie	Solvent	Ligand[b]	pH range	$\Delta\varepsilon$ M^{-1} x 10^{-4} cm^{-1}
1-104	H_2O	MET-Fe-HIS	2.0-7.0	11.5
	9 M urea	X-Fe-HIS	2.7-11.2	11.0
HP 11-21	H_2O	X-Fe-HIS	2.0-10.3	10.0
		Fe-HIS	2.0-5.6	6.4
		X-Fe-HIS	5.6-10.3	3.6
	0.5 M KCl	X-Fe-HIS	2.0-9.9	9.1
		Fe-HIS	2.0-4.8	4.4
		X-Fe-HIS	4.8-9.9	4.7
HP 14-21	H_2O	Fe-HIS	2.0-5.4	4.8
	0.1 M imidazole	HIS-Fe-HIS	2.0-7.0	7.6
		HIS-Fe-HIS	7.0	3.8[c]
	0.1 M MET[d]	MET-Fe-HIS	7.0	3.6[c]
	0.1 M imidazole 9 M urea	HIS-Fe-HIS	3.0-7.3	6.8
$(CM\ MET)_2$	H_2O	CM MET-Fe-HIS	2.2-7.2	10.0
		Fe-HIS	2.2-4.0	6.0
		CM MET-Fe-HIS	4.0-7.2	4.0

[a]Values obtained upon titration of each specie in the indicated pH range.

[b]The ligand(s) forming a new coordinate-covalent bond in the indicated pH range is (are) underlined. X, ligand not known, see text.

[c]Obtained upon addition of ligand at constant pH.

[d]N-acetyl-methionine.

Figure 11. Reverse spectrophotometric titrations of cytochrome c hemopeptides. A, HP 11-21. (O) Absorbance at 395 nm in the absence of extrinsic salt, (●) absorbance at 395 nm in the presence of 0.5 M NaCl. B, HP 14-21. (O) Absorbance at 395 nm in the absence of extrinsic salt, (▲) absorbance at 395 nm in the presence of 0.1 M imidazole, (△) carboxymethylated HP 14-21, absorbance at 390 nm.

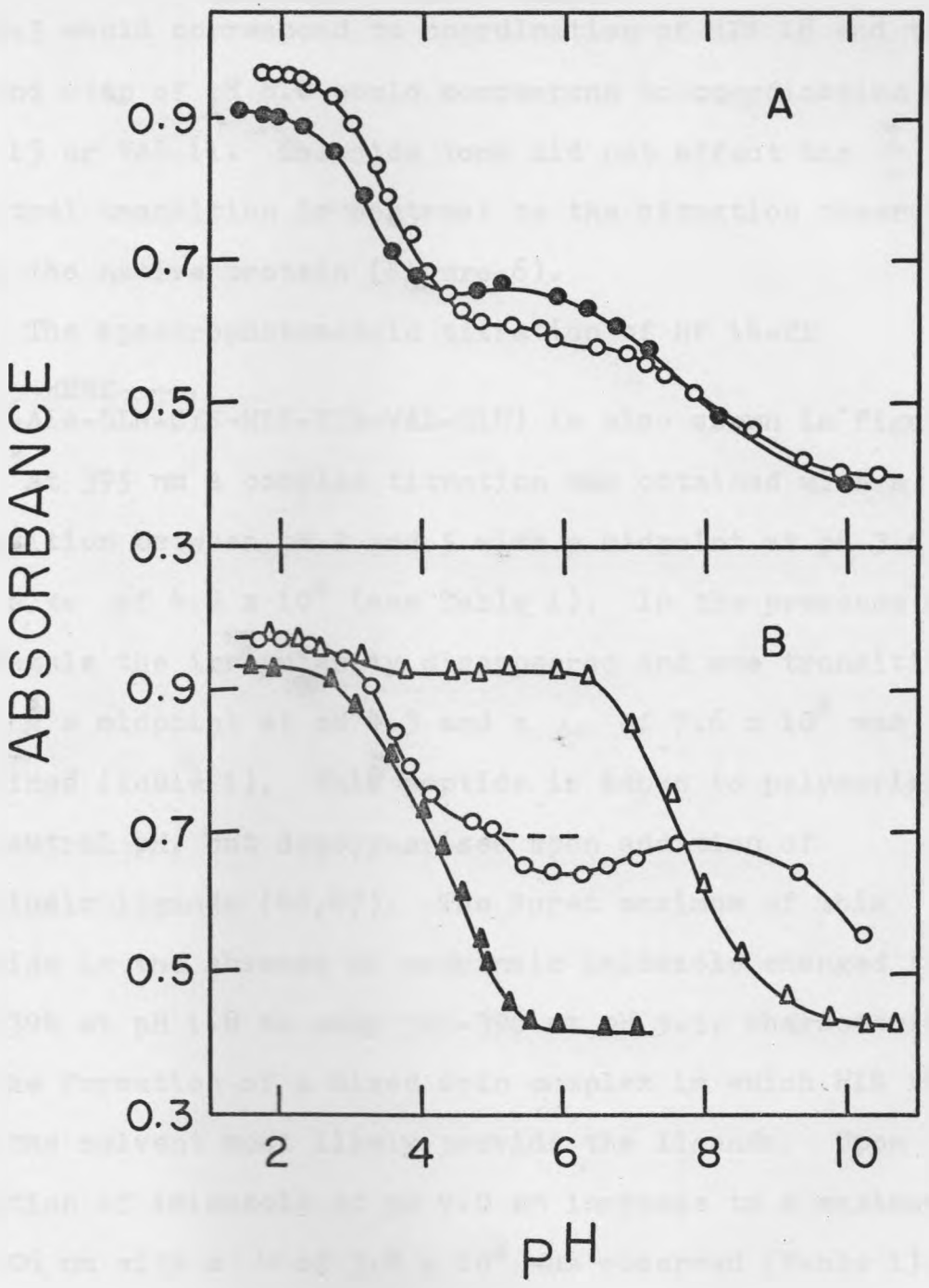

Figure 11

pK 3.5 would correspond to coordination of HIS 18 and the second step of pK 8.0 would correspond to coordination of LYS 13 or VAL 11. Chloride ions did not affect the spectral transition in contrast to the situation observed with the native protein (Figure 6).

The spectrophotometric titration of HP 14-21 (CYS-ALA-GLN-CYS-HIS-THR-VAL-GLU) is also shown in Figure 11. At 395 nm a complex titration was obtained with a transition between pH 2 and 5 with a midpoint at pH 3.6 and a $\Delta\varepsilon$ of 4.8×10^4 (see Table 1). In the presence of imidazole the irregularity disappeared and one transition having a midpoint at pH 4.3 and a $\Delta\varepsilon$ of 7.6×10^4 was obtained (Table 1). This peptide is known to polymerize at neutral pH, but depolymerizes upon addition of extrinsic ligands (46,47). The Soret maximum of this peptide in the absence of extrinsic imidazole changed from 393-394 at pH 1.8 to only 395-396 at pH 5.5, characteristic of the formation of a mixed spin complex in which HIS 18 and the solvent most likely provide the ligands. Upon addition of imidazole at pH 7.0 an increase to a maximum at 406 nm with a $\Delta\varepsilon$ of 3.8×10^4 was observed (Table 1). This value of 406 nm is characteristic of a low spin complex where the ligand in the fifth position is provided by the peptide, HIS 18, and an extrinsic ligand is coordinated in the sixth position. This interpretation

is supported by the observation that HP 14-21 has a high spin band around 620 nm throughout the pH range studied, which disappears in the presence of imidazole at pH values greater than 5.9. Addition of N-acetyl methionine at pH 7.0 also shifts the Soret maximum to 406 nm. A $\Delta\varepsilon$ of 3.6×10^4 accompanied the shift (Table 1).

Figure 11 also shows the titration of HP 14-21 in which HIS 18 is carboxymethylated. The transition occurring between pH 2 and 5 with the unmodified peptide is now absent, while a new transition having a midpoint at pH 7.7 is observed. This indicates that the transition having an apparent pK of 3.5-3.6 observed with HP 11-21 and HP 14-21 is due to coordination of HIS 18.

D. Observed $\Delta\varepsilon_{395}$ for Ligand Coordination and Heme Perturbation

The $\Delta\varepsilon$ at 395 nm calculated from the titration curves of HP 11-21, HP 14-21, and $(CM\ MET)_2$ cytochrome c, interpreted here as being due to ligand coordination are summarized in Table 1. Coordination of HIS 18 in the fifth position produced an average $\Delta\varepsilon$ of 5.4×10^4 with a mean absolute deviation of $\pm$ 1.0×10^4. The $\Delta\varepsilon$ expected for coordination of a ligand in the sixth position was estimated from the titration curves of model compounds that provide both ligands, such as HP 11-21 and $(CM\ MET)_2$, and by addition of the ligands imidazole and N-acetyl

methionine to HP 14-21. An average $\Delta\varepsilon$ of 3.9×10^4 with a mean absolute deviation of $\pm$ 0.3×10^4 was obtained.

The absorbance changes due to changes in heme environment were estimated by solvent perturbation difference spectra of HP 11-21 and HP 14-21 in the presence of imidazole (see Methods). Using ethylene glycol as a perturbing agent, $\Delta\varepsilon$ values of 0.23×10^4 and 0.11×10^4 were observed at 395 nm for HP 14-21 and HP 11-21 respectively.

E. Other Denaturants

The Soret maximum of cytochrome c changes from 410 nm in the absence of urea to 407 nm in the presence of 9 M urea, a concentration that unfolds the polypeptide chain. The effect of the denaturants urea and GuHCl at neutral pH on the extinction at 402 nm is shown in Figure 12. Single step transitions with midpoints at 2.6 and 6.3 M were found for GuHCl and urea, respectively. The reversibility of this transition was investigated by placing cytochrome c in high concentration of denaturing agent and then diluting to a lower concentration. The absorbance changes were found to be reversible. The 695 nm absorbance band decreased with increasing concentrations of GuHCl (Figure 12) but the 620 nm band was found to be absent in 9M urea or 6M GuHCl. The absence of a high spin band together with a Soret maximum of 407

Figure 12. Absorbance changes of cytochrome c in denaturing solutions at pH 7.0. Absorbance at 402 nm (●) and 695 nm (○) in 0.1 M phosphate pH 7.0 as a function of the concentration of Gu HCl. Protein concentrations and $\Delta\varepsilon$'s: 402 nm, 1.6×10^{-5} M and 2.6×10^{4}; 695 nm, 7.0×10^{-4} M and 1.1×10^{3}. Absorbance at 402 nm (△) in 0.02 M phosphate as a function of the concentration of urea. Protein concentration, 1.5×10^{-5} M; $\Delta\varepsilon$, 2.5×10^{4}.

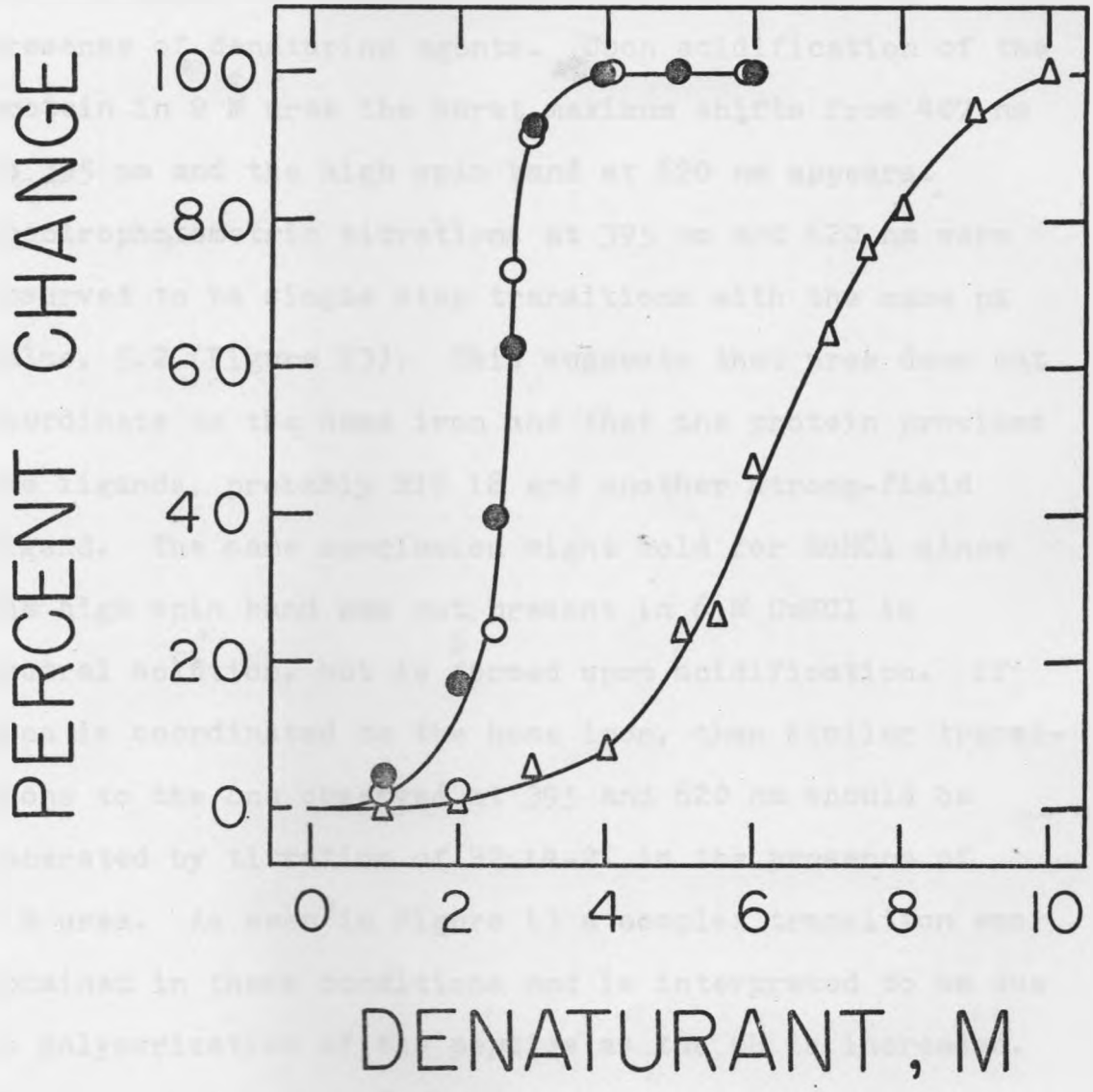

Figure 12

nm indicates the presence of a low spin complex. The ligands forming the low spin complex are provided by the protein, by urea or both. Evidence favoring the first possibility is provided by titration studies in the presence of denaturing agents. Upon acidification of the protein in 9 M urea the Soret maximum shifts from 407 nm to 395 nm and the high spin band at 620 nm appears. Spectrophotometric titrations at 395 nm and 620 nm were observed to be single step transitions with the same pK value, 5.2 (Figure 13). This suggests that urea does not coordinate to the heme iron and that the protein provides the ligands, probably HIS 18 and another strong-field ligand. The same conclusion might hold for GuHCl since the high spin band was not present in 6 M GuHCl in neutral solution, but is formed upon acidification. If urea is coordinated to the heme iron, then similar transitions to the one observed at 395 and 620 nm should be generated by titration of HP 14-21 in the presence of 9 M urea. As seen in Figure 13 a complex transition was obtained in these conditions and is interpreted to be due to polymerization of the peptide as the pH is increased. This is based on the fact that the spectrum of the peptide changed to that characteristic of a polymer upon increasing pH, as shown in Figure 14. In the presence of 9 M urea over the pH range 3-12, HP 14-21 has a Soret maximum below 400 nm and shifted to 408 nm upon adition of

Figure 13. Spectrophotometric titrations of cytochrome c and HP 14-21 in the presence of 9 M urea. Cytochrome c, absorbance at 620 (0) and at 395 nm (●). HP 14-21, absorbance at 395 nm (▲) and absorbance at 395 nm also in the presence of 0.1 M imidazole (△).

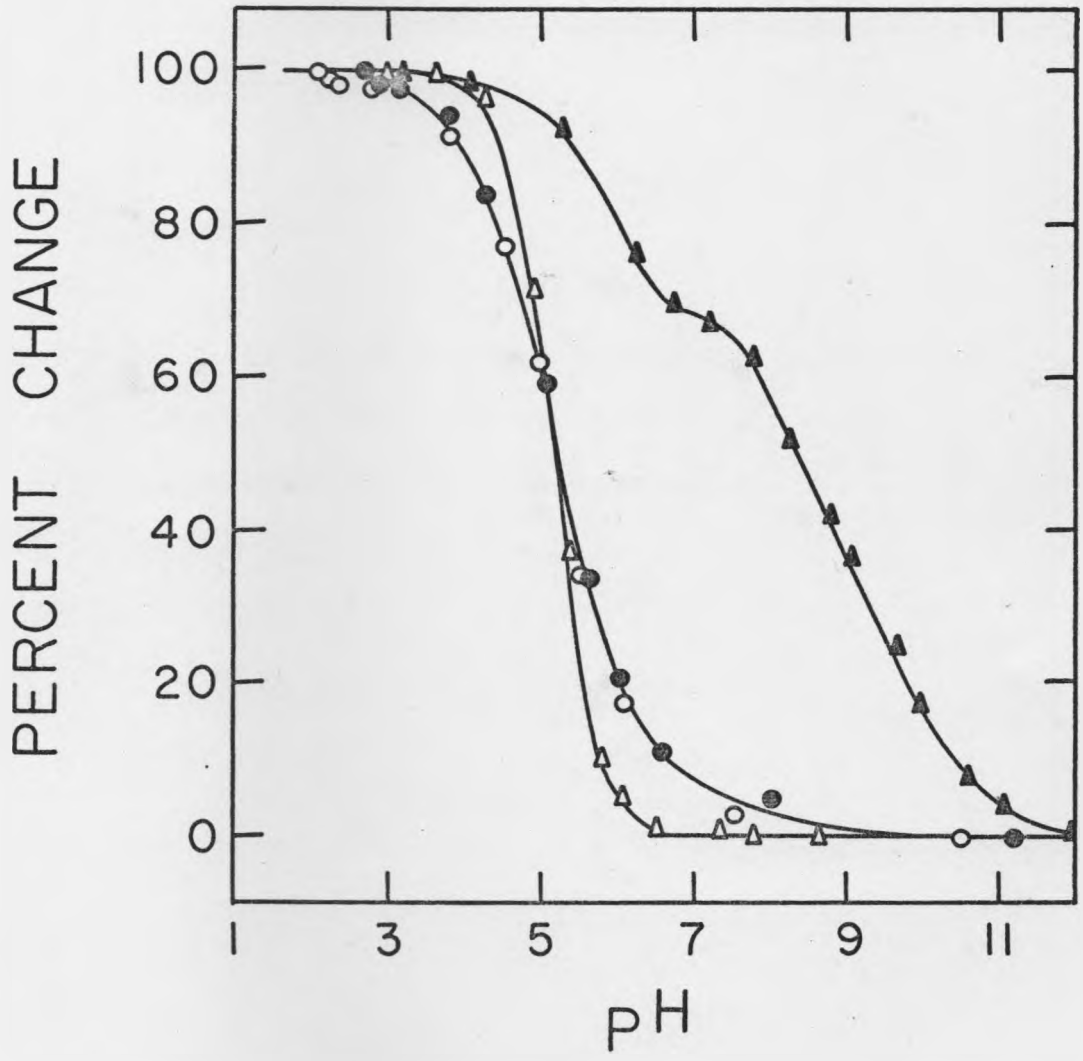

Figure 14

Figure 14. Absorption spectra of HP 14-21 in 9 M urea. A, pH 8.8; B, pH 11.9; C, pH 11.9 also in the presence of 0.1 M imidazole.

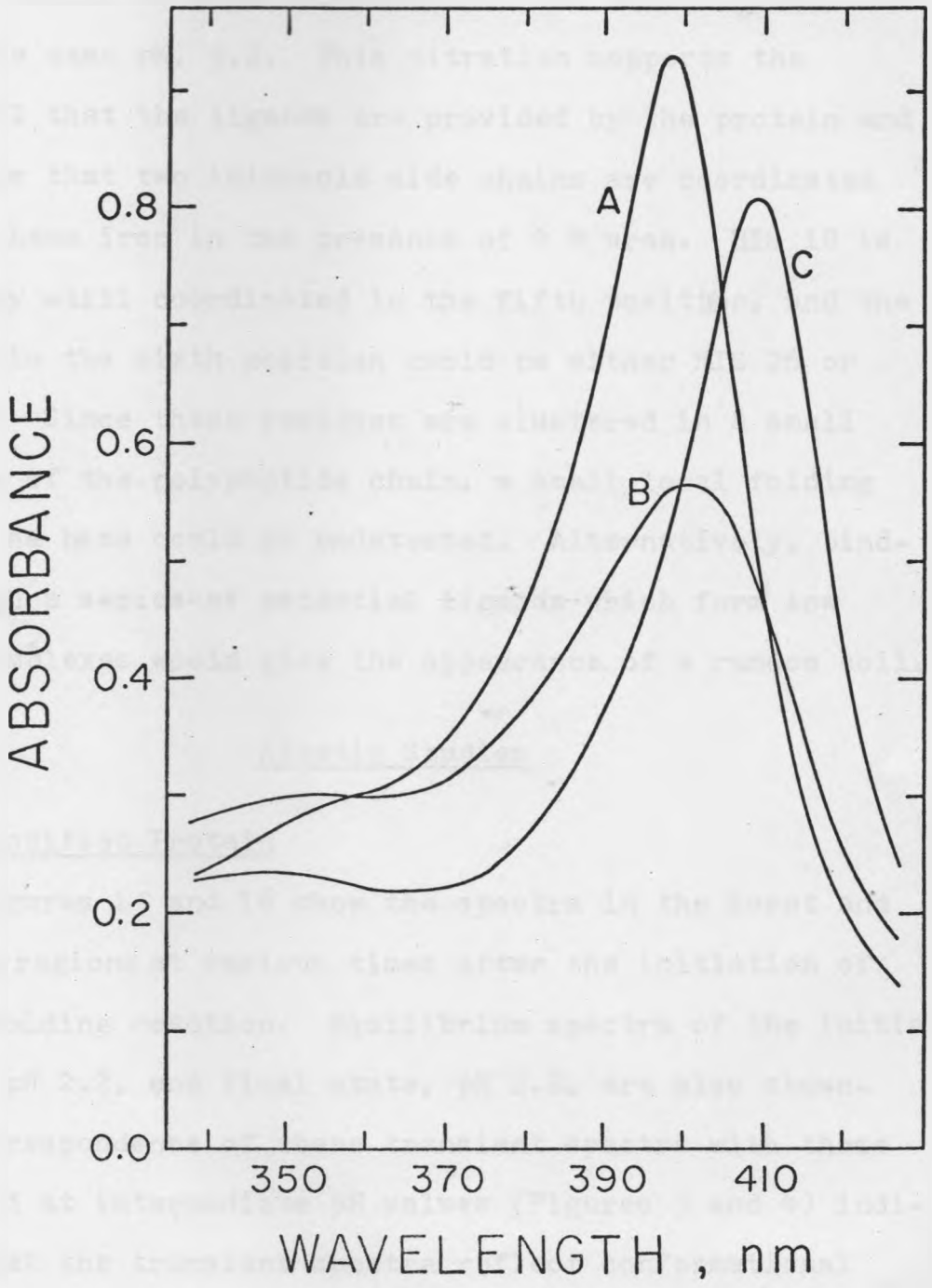

Figure 14

imidazole (Figure 14). Titration of the peptide in the presence of 9 M urea and 0.1 M imidazole gives a transition curve similar to that obtained with the whole protein and with the same pK, 5.2. This titration supports the proposal that the ligands are provided by the protein and suggests that two imidazole side chains are coordinated to the heme iron in the presence of 9 M urea. HIS 18 is possibly still coordinated in the fifth position, and the ligand in the sixth position could be either HIS 26 or HIS 33. Since these residues are clustered in a small portion of the polypeptide chain, a small local folding about the heme could go undetected. Alternatively, binding with a series of potential ligands which form low spin complexes would give the appearance of a random coil.

Kinetic Studies

A. Unmodified Protein

Figures 15 and 16 show the spectra in the Soret and visible regions at various times after the initiation of the refolding reaction. Equilibrium spectra of the initial state, pH 2.2, and final state, pH 2.8, are also shown. The correspondence of these transient spectra with those observed at intermediate pH values (Figures 3 and 4) indicate that the transient spectra reflect conformational changes, since the refolding, as evidenced by reduced viscosity changes, correlates closely with spectral

Figure 15. Refolding of cytochrome c: spectra in the Soret region at various times after mixing for a pH jump 2.2-2.8 at 25°. A, initial spectrum; B, 10 msec; C, 40 msec; D, 100 msec; E, 160 msec; F, final spectrum. Concentration, 2.0×10^{-6} M. See Methods.

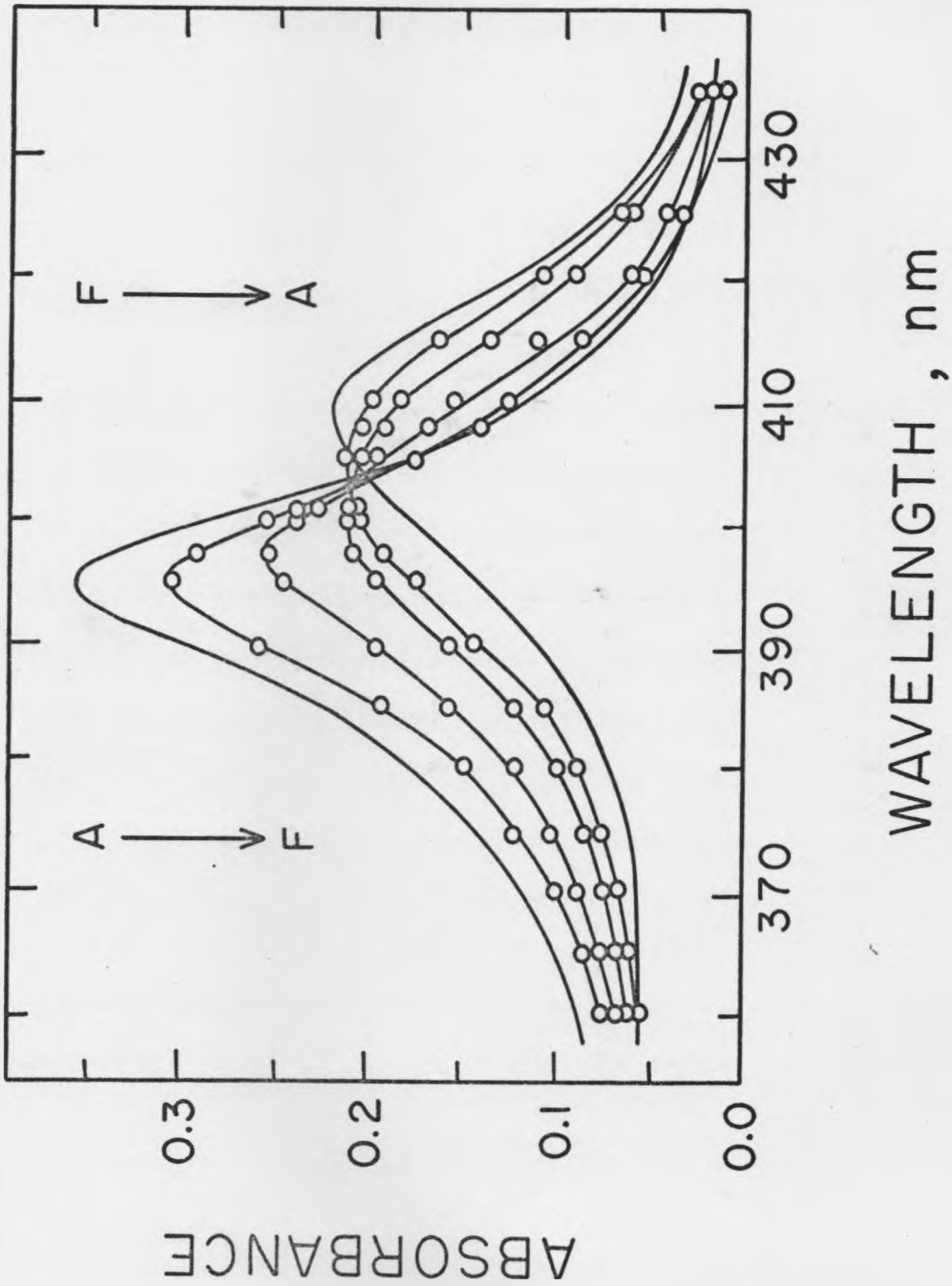

Figure 15

Figure 16. Refolding of cytochrome c: spectra in the visible region at various times after mixing for a pH jump 2.2-2.8 at 25°. A, initial spectrum; B, 10 msec; c, 20 msec; D, 40 msec; E, 80 msec; F, 500 msec; G, final spectrum. Concentration, 11 x 10^{-5} M.

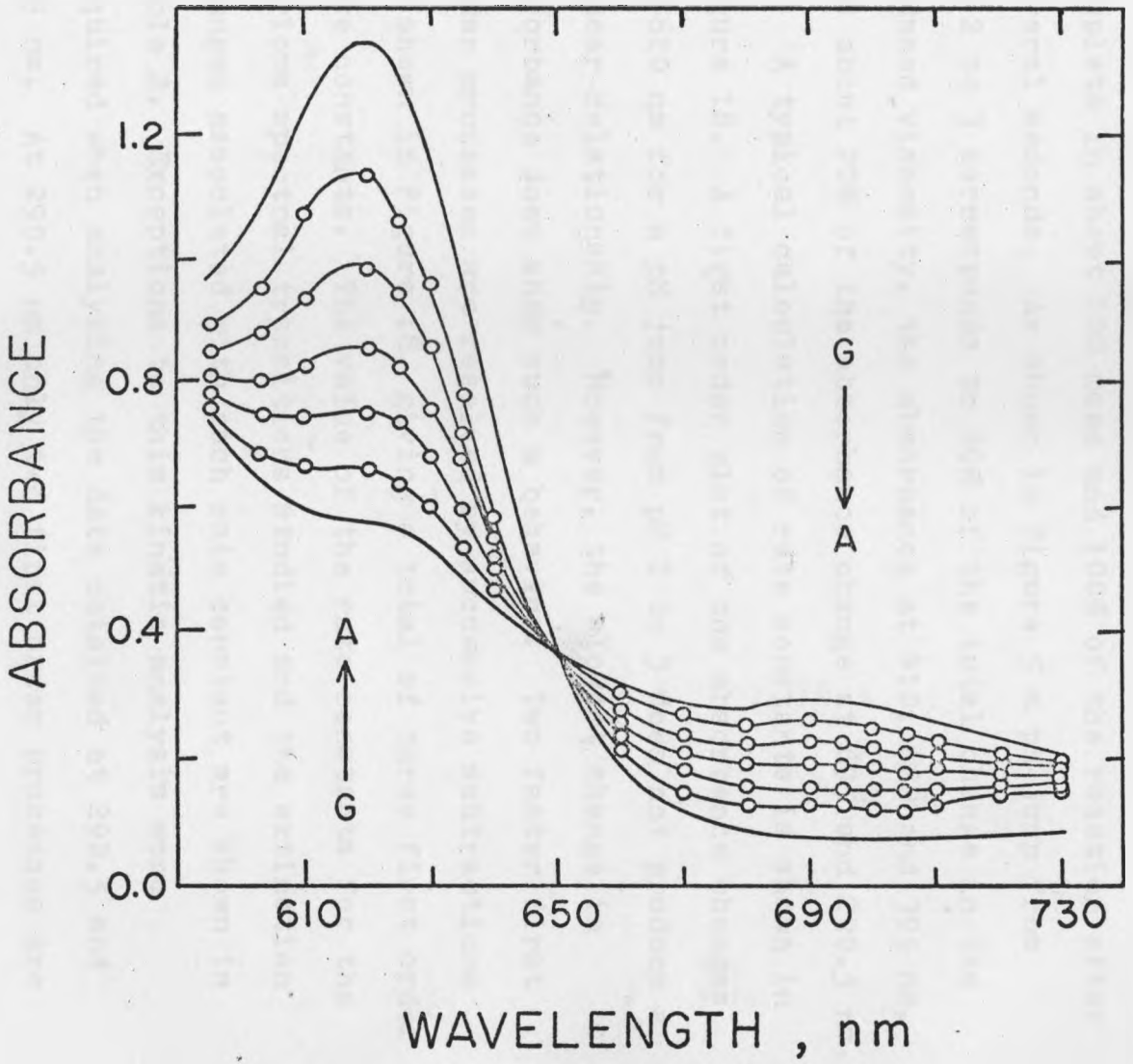

Figure 16

parameters (Figure 5).

Figure 17 compares the rate of absorbance change at different wavelengths for a pH jump from pH 2 to 3. For all transitions, approximately 80% of the reaction is complete in about 100 msec and 100% of the reaction after several seconds. As shown in Figure 5 a pH jump from pH 2 to 3 corresponds to 90% of the total change in the reduced viscosity, the absorbance at 410, 620 and 395 nm, and about 70% of the absorbance change at 695 and 290.5 nm.

A typical calculation of rate constants is shown in Figure 18. A first order plot of the absorbance changes at 620 nm for a pH jump from pH 2 to 3 does not produce a linear relationship. However, the slowest change in absorbance does show such a behavior. Two faster first order processes are resolved by successive subtractions as shown in Figure 18, giving a total of three first order rate constants. The value of the rate constants for the various spectral transitions studied and the extinction changes associated with each rate constant are shown in Table 2. Exceptions to this kinetic analysis were required when analyzing the data obtained at 290.5 and 695 nm. At 290.5 nm only two first order processes are observed and the corresponding rate constants were calculated without subtraction of the slower absorbance changes from the observed values since this resulted in a non-linear relationship. The same procedure was used for the

Figure 17. Refolding of cytochrome c: absorbance at several wavelengths as a function of time for a pH jump 2.0-3.0 at 25°. (▲) 695 nm, (○) 620 nm, (●) 410 nm, (△) 395 nm, (□) 290.5 nm.

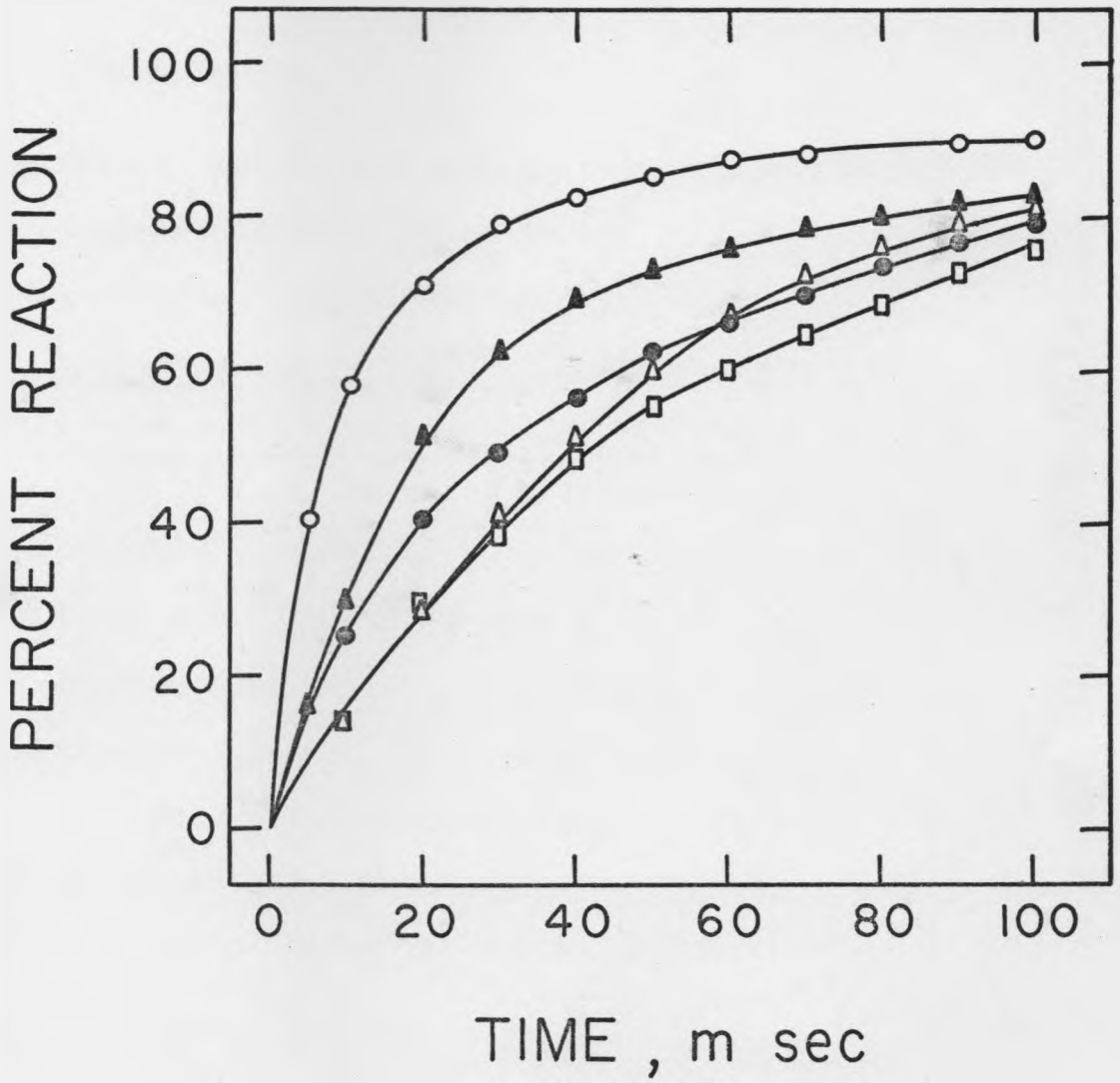

Figure 17

Figure 18. Calculation of rate constants. First order plot of the refolding reaction measured at 620 nm for a pH jump 2.0-3.0 at 25°. (●) Absorbance difference as a function of time, (0) values obtained after subtraction of the initial value of curve II from curve I, (△) values obtained after subtraction of curve IV from curve III. The values of k_1, k_2, and k_3 were calculated from the slopes of lines V, IV, and II respectively. The $\Delta\varepsilon$ value associated with each rate process was calculated from the intersection of these lines with the ordinate.

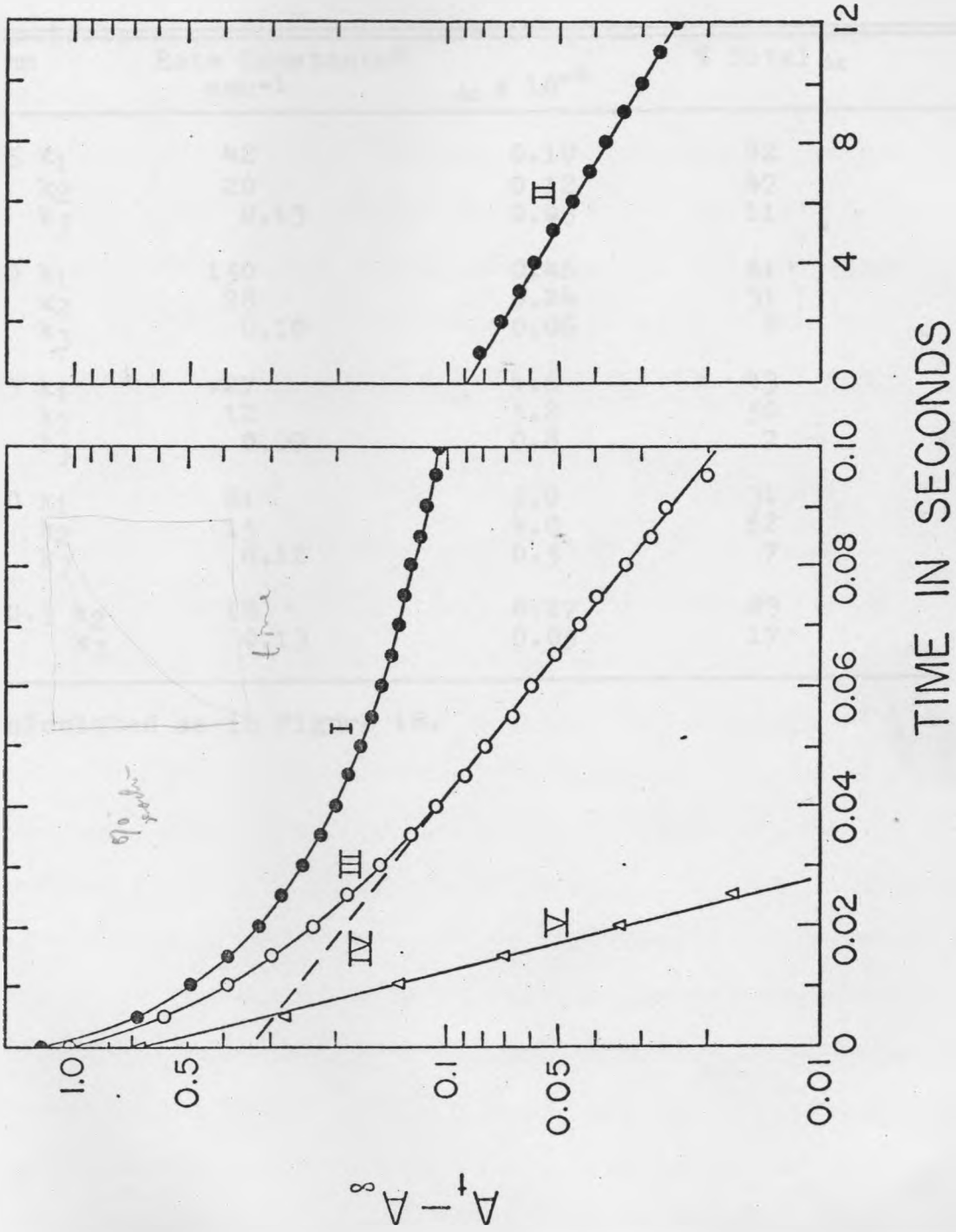
1.0
0.5
0.1
0.05
0.01
0
0.02
0.04
0.06
0.08
0.10
0
4
8
12
I
II
III
IV
V
$A_t - A_\infty$
TIME IN SECONDS

Figure 18

TABLE 2

Refolding of Cytochrome c Measured at Several Wavelengths. pH Jump 2.0-3.0, 25°

nm	Rate Constants[a] sec^{-1}	$\Delta\varepsilon \times 10^{-4}$	% Total $\Delta\varepsilon$
695 k_1	42	0.10	42
k_2	20	0.12	47
k_3	0.13	0.03	11
620 k_1	150	0.46	61
k_2	28	0.24	31
k_3	0.10	0.06	8
395 k_1	27	4.6	43
k_2	12	5.2	50
k_3	0.09	0.8	7
410 k_1	81	2.0	31
k_2	15	4.0	62
k_3	0.12	0.5	7
290.5 k_2	18	0.27	83
k_3	0.13	0.05	17

[a]Calculated as in Figure 18.

calculation of k_1 at 695 nm.

The effect of the final pH on the refolding reaction was also studied. Figure 19 shows the effect of pH on k_1 and k_2 for the 395 nm transition at 5°. At pH values greater than 4, k_1 was estimated by extrapolation of the experimental points to the starting absorbance. Between pH 4 and 6 the experimental points, after subtraction of the absorbance values associated with k_2, did not extrapolate to the initial absorbance value so k_1 could not be estimated. The rate constant k_3 has a value of 0.033 ± 0.02 sec^{-1} in the range studied.

The effect of the final pH on the various rate constants for the 620 nm transition at 25° is shown in Figure 20. From pH 2.7 to 3.5 the rate constants were calculated as in Figure 18. At pH 4.15, after the initial decrease in absorbance, a relatively fast increase is observed after which the final absorbance value is slowly attained. This situation is shown in Figure 21, and is observed up to a final pH value of 6.5. Two rate constants are associated with the fast initial absorbance decrease accounting for almost 100% of the absorbance change at 620 nm. As seen in Figure 21, more than 80% of the change in absorbance occurs within 6 msec, the first experimental point. A similar situation is observed in the pH range 4.15-7.5. For this reason, at pH 4.15 or above, only rough estimates of k_1 are made, by extrapolation of the

Figure 19. The effect of pH on the refolding reactions measured at 395 nm, 5°. The rate constants k_1 (●) and k_2 (○) calculated, as described in Figure 18, at the indicated pH values. Concentration: 6.2×10^{-6} M. See text and Methods.

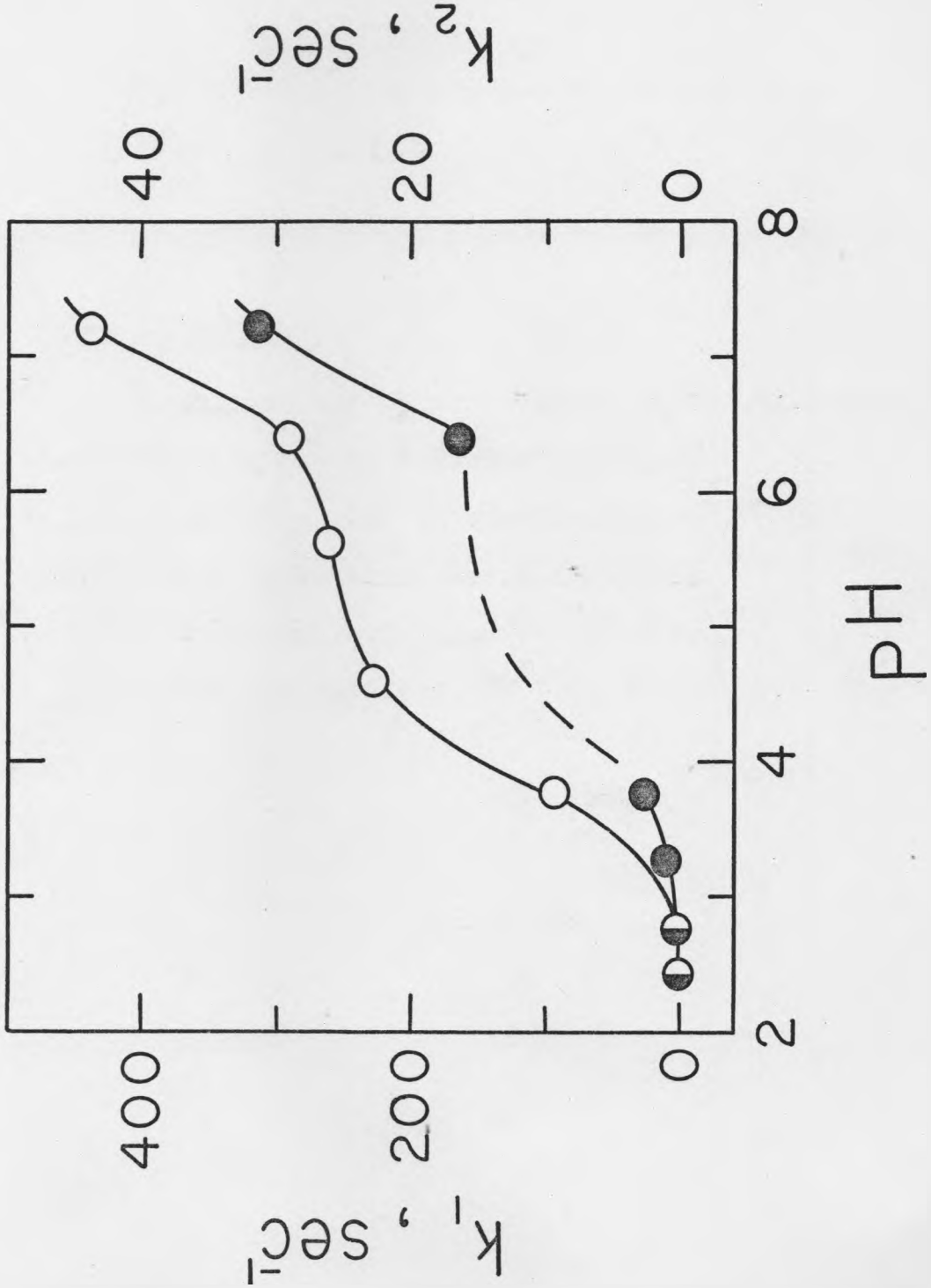

Figure 19

Figure 20. The effect of pH on the refolding reactions measured at 620 nm, 25°. The rate constants k_1 (●), k_2 (○), k_{-2} (△) and k_3 (▲) were determined at the indicated pH values. Concentration: 1.5×10^{-4} M. See text and Methods.

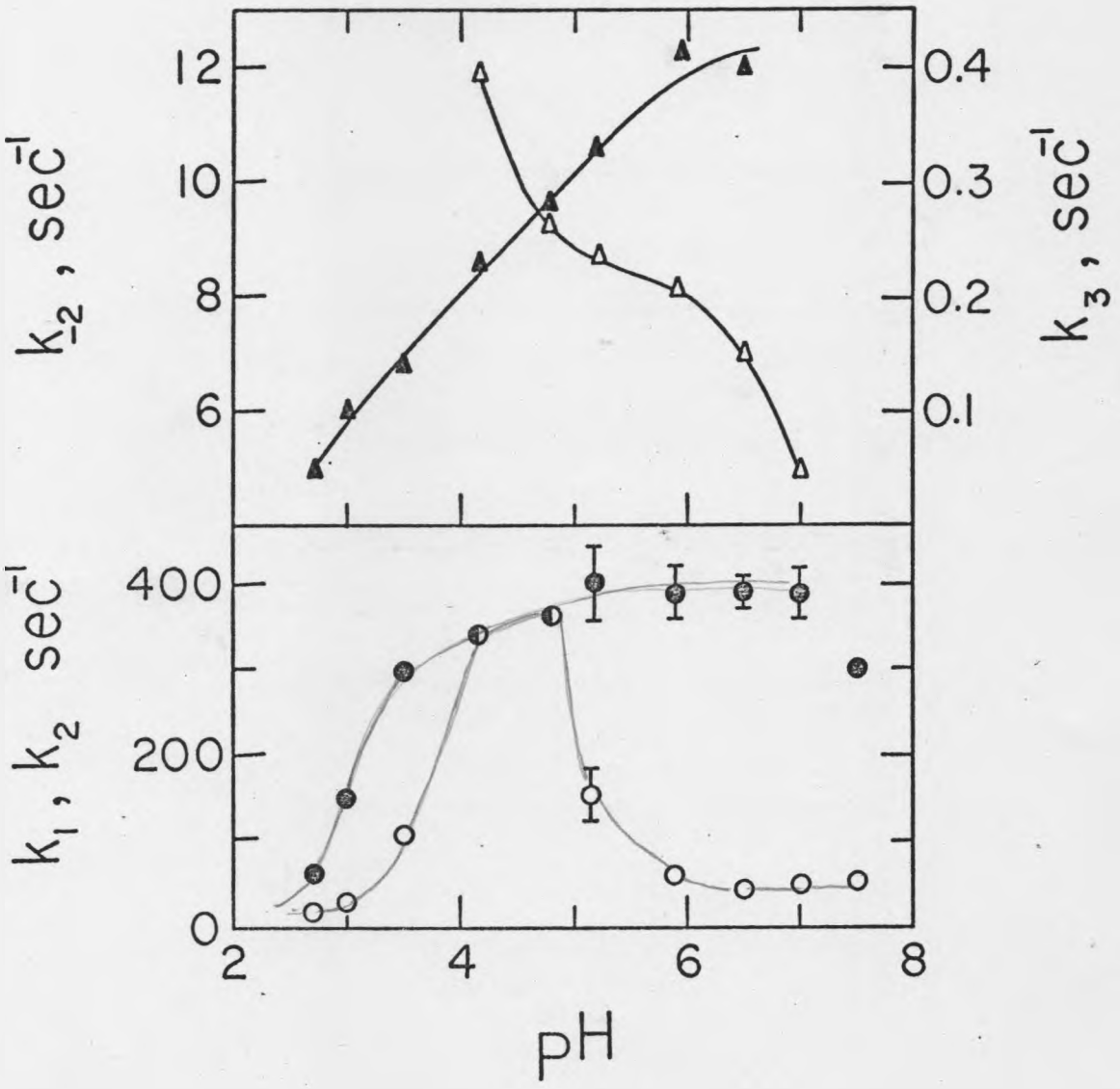

Figure 20

Figure 21. Decrease in absorbance at 620 nm during refolding of cytochrome c. pH jump 2.0-4.15 at 25°.

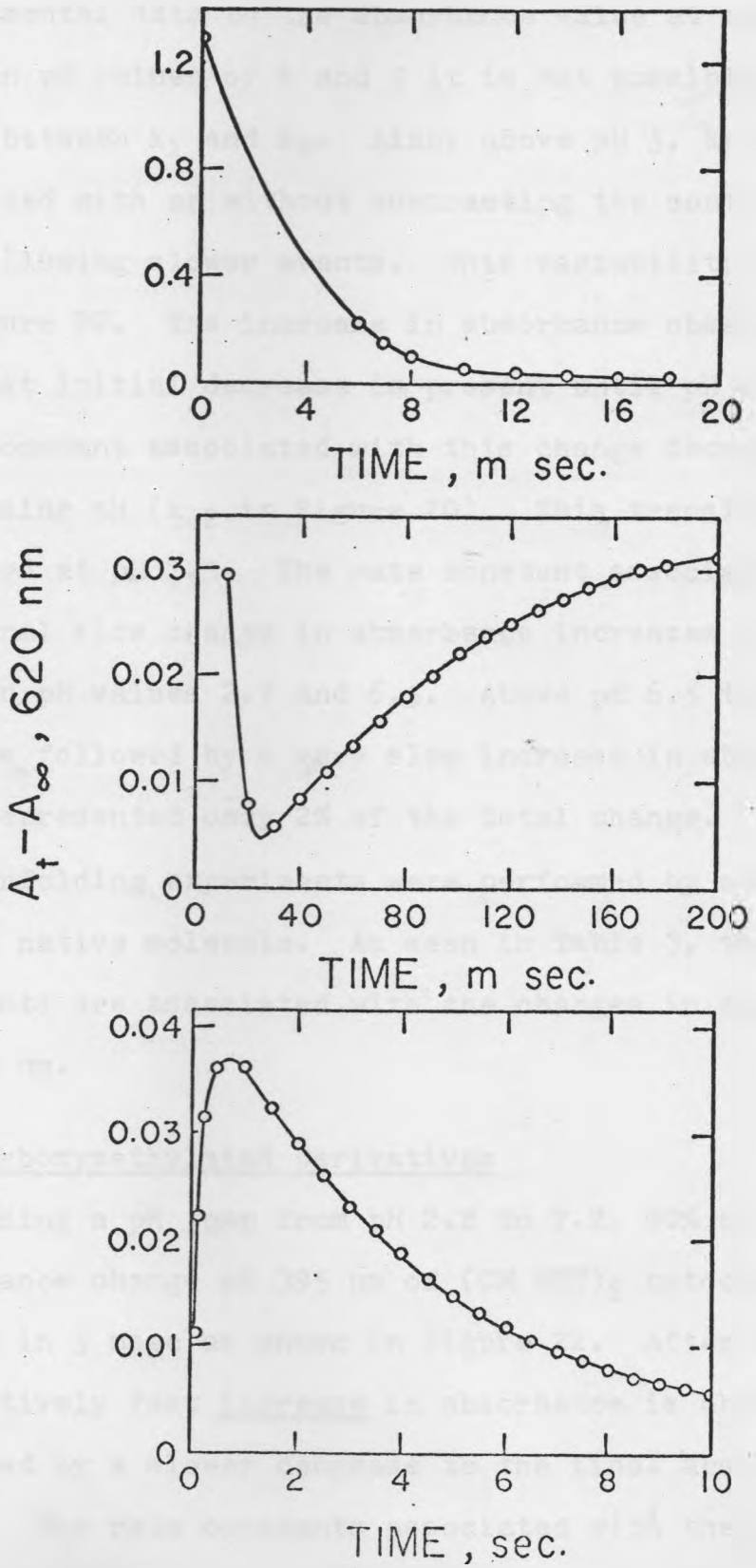
1.2
0.8
0.4
0
0
4
8
12
16
20
TIME , m sec.
$A_t - A_\infty$, 620 nm
0.03
0.02
0.01
0
0
40
80
120
160
200
TIME , m sec.
0.04
0.03
0.02
0.01
0
0
2
4
6
8
10
TIME , sec.

Figure 21

experimental data to the absorbance value at zero time. Between pH values of 4 and 5 it is not possible to distinguish between k_1 and k_2. Also, above pH 5, k_1 could be estimated with or without subtracting the contribution of the following slower events. This variability is shown in Figure 20. The increase in absorbance observed after the fast initial decrease is present until pH 7, and the rate constant associated with this change decreases with increasing pH (k_{-2} in Figure 20). This transition is not observed at pH 7.5. The rate constant associated with the final slow change in absorbance increases about 8-fold between pH values 2.7 and 6.5. Above pH 6.5 this transition is followed by a very slow increase in absorbance that represented only 2% of the total change.

Unfolding experiments were performed by acidification of the native molecule. As seen in Table 3, three rate constants are associated with the changes in absorbance at 395 nm.

B. Carboxymethylated Derivatives

Using a pH jump from pH 2.2 to 7.2, 90% of the total absorbance change at 395 nm of $(CM\ MET)_2$ cytochrome c occurs in 5 msec as shown in Figure 22. After this time a relatively fast increase in absorbance is observed, followed by a slower decrease to the final absorbance value. The rate constants associated with these

TABLE 3

Unfolding of Cytochrome c. pH Jump 6.1-1.9, 4°

Rate Constants[a] sec^{-1}		$\Delta\varepsilon_{395} \times 10^{-4}$ $M^{-1}cm^{-1}$
k_1	[b]	1.8
k_2	0.47	8.5
k_3	0.008	1.1

[a]Calculated as in Figure 18.

[b]Too fast to measure.

Figure 22. Decrease in absorbance at 395 nm during refolding of $(CM\ MET)_2$ cytochrome *c*. pH jump 2.2-7.2 at 25°. See text.

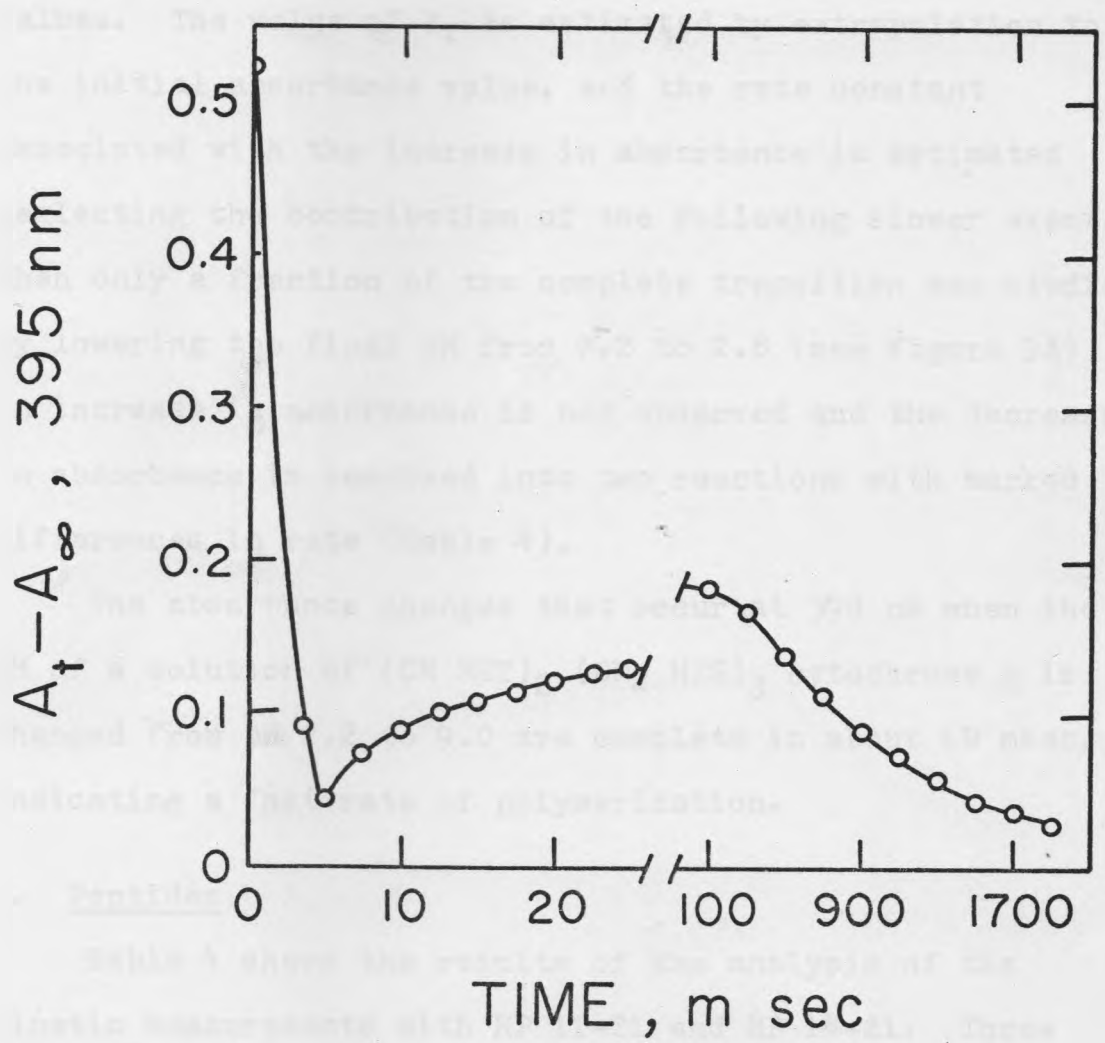

Figure 22

transitions and their respective $\Delta\varepsilon$ values are shown in Table 4. Since the final absorbance value of the first two transitions can not be estimated, the value of the rate constants associated with them are only approximate values. The value of k_1 is estimated by extrapolation to the initial absorbance value, and the rate constant associated with the increase in absorbance is estimated neglecting the contribution of the following slower event. When only a fraction of the complete transition was studied by lowering the final pH from 7.2 to 2.8 (see Figure 9A) an increase in absorbance is not observed and the decrease in absorbance is resolved into two reactions with marked differences in rate (Table 4).

The absorbance changes that occur at 390 nm when the pH of a solution of $(CM\ MET)_2$ $(CM_2\ HIS)_3$ cytochrome c is changed from pH 2.2 to 9.0 are complete in about 10 msec, indicating a fast rate of polymerization.

C. Peptides

Table 4 shows the results of the analysis of the kinetic measurements with HP 11-21 and HP 14-21. Three different pH jumps were studied with HP 11-21. Four first order rate constants are observed in a pH jump that comprises the total transition observed in the equilibrium titration (Figure 11), with the initial reaction rate being too fast to measure. One rate constant and three

TABLE 4

$\Delta\varepsilon_{395}$ Associated with the Refolding Reactions[a]

Specie	pH Jump	t°	Rate Constants[b] sec^{-1}	$\Delta\varepsilon^{c} \times 10^{-4}$ M^{-1}cm^{-1}
1-104	2 to 3.2-7.2 (6 measurements)	5°	k_1 10-310	6.8 ± 1.9
			k_2 2.7-44	4.0 ± 1.8
			k_3 0.033 ± 0.021	0.32 ± 0.10
	2 to 4.7-7.5 (2 measurements)	25°	k_1 290 ± 50	8.4 ± 0.4
			k_2 75 ± 15	2.0 ± 0.2
			k_3 0.23 ± 0.06	0.6 ± 0.1
$(CM\ MET)_2$	2.2 to 2.8	25°	k_1 28	1.3
			k_2 2.1	0.36
	2.2 to 7.2	25°	k_1 } 500	8.7
			k_2	
			k_2 40	-2.5
			k_3 1.1	4.3
HP 11-21	2.0 to 9.9	25°	k_1 [d]	4.2
			k_2 32	2.5
			k_3 6.8	2.0
			k_4 0.22	0.33
	2.0 to 5.2		k_1 11	3.9
	5.3 to 9.9		k_2 46	1.8
			k_3 6.8	1.9
			k_4 0.19	0.23
HP 14-21	2.0 to 7.0	25°	k_1 [d]	3.4
			k_2 31	1.5

[a]Procedures are described under Material and Methods.

[b]Calculated as in Figure 18.

[c]Mean absolute deviations are shown.

[d]Too fast to measure.

rate constants are associated with pH jumps corresponding to the first and second steps observed in the equilibrium titration, respectively. The rate constants for these transitions and the corresponding observed $\Delta\varepsilon$ values are shown in Table 4.

D. Other Denaturants

The changes in absorbance at 402 nm accompanying dilution of a solution of cytochrome c in 8 M urea to 4 M urea or dilution of a solution of cytochrome c in 3.5 M GuHCl to 1.75 M were followed in the stopped-flow apparatus. As seen in Table 5, the changes in absorbance in both cases could be analyzed in terms of three first order processes.

TABLE 5

Kinetics of Spectral Changes in Urea and Gu HCl at pH 7.0, 25°

Denaturant	Rate Constants[a] sec^{-1}		$\Delta\varepsilon \times 10^{-4}$ $M^{-1}cm^{-1}$ 402
Urea (8 → 4 M)	k_1	4.3	0.50
	k_2	1.3	0.50
	k_3	0.36	0.47
Gu HCl (3.5 → 1.75 M)	k_1	8.1	0.50
	k_2	2.1	0.64
	k_3	0.68	0.33

[a]Calculated as in Figure 18. See Methods.

DISCUSSION

Reversibility

The viscosity and spectral properties of cytochrome c and of the model compounds used in this work indicate that the protein can be extensively unfolded by acidification, and that its native structure can be quantitatively recovered by neutralization. This indicates that refolding is a very specific process whereby the amino acid residues interact in a unique way to form the structure characteristic of the native protein. The heme moiety probably plays a fundamental role in maintaining this structure since apocytochrome c has the properties characteristic of a random coil (48). The reversibility of the unfolding transition is in agreement with the pH stability of cytochrome c as measured by its catalytic activity in the succinate oxidase system. The protein was found to be perfectly stable to preincubation from pH 1.6 to 12.3 (49).

When studying the reversibility of protein unfolding it is important to be certain that the unfolded state is devoid of any specific structure. In this respect, it is of importance that the coordinate covalent bonds between the polypeptide and the heme iron are cleaved at

low pH. This is not the situation when urea or GuHCl are used as denaturing agents, where spectral measurements indicate that the protein still provides both ligands in the fifth and sixth coordination position. The spectral properties of cytochrome c and HP 14-21 in urea do not agree with the conclusion that urea replaces the intrinsic ligands (28,50) and the pK of the acid transition suggests two imidazole side chains, HIS 18 and HIS 26 or 33, are coordinated to the heme iron in urea or GuHCl.

Importance of Ligand Coordination in the Formation of the Native Structure

Several chemically modified cytochromes c have been employed to examine the structure and function of the protein (51). In this study two derivatives in which the fifth, and the sixth ligand to the heme iron are modified were examined. The spectral and viscosity data indicate that $(CM\ MET)_2$ cytochrome c is still able to fold into a structure that is similar to that of the native protein. However, this derivative is less resistant to the unfolding action of acid as evidenced by the higher pK of the spectral and viscosity transitions (Figure 9), it does not present a 695 nm band, and does not possess electron transfer activity (41). Although MET 80 cannot be selectively modified, MET 65 can be selectively modified. The ORD spectrum of $(CM\ MET_{65})$ cytochrome c is very similar

to that of the native protein (38) indicating little structural alteration. By the X-ray analysis of crystalline cytochrome c (24), MET 65 was found to be in the surface of the molecule so little change in the protein's properties are to be expected from its modification. These observations indicate that the changes in properties of the protein observed upon modification of MET 65 and MET 80 can be attributed to be due to modification of the latter.

The observed properties of (CM MET)$_2$ (CM$_2$ HIS)$_3$ cytochrome c indicate that this derivative retains little if any of the conformation of the native protein. Unfortunately, the two natural ligands, HIS 18 and MET 80, cannot be selectively modified. Carboxymethylation of HIS 18 is accompanied by carboxymethylation of MET 65 and 80, HIS 26 and 33, and two other residues, identified as ε-monocarboxymethyl lysine (see Methods). Modification of MET 65 (38), MET 65 and 80 (38,42), MET 65 and 80 and one of HIS (39), probably HIS 33 (40), does not seem to alter the conformation of the protein to any significant extent. Guanidination of all 19 lysines of cytochrome c does not alter the properties of the protein (52). Modification of all lysines and one methionine of pseudomonas cytochrome c results in the retention of the spectral characteristics of the native molecule (53). Thus the

drastic changes in the properties of the protein accompanying formation of (CM MET)$_2$ (CM$_2$ HIS)$_3$ can be ascribed by difference to HIS 18, HIS 26 or both HIS 18 and 26. HIS 18, the ligand in the fifth position must play an important role in the structure of the protein. In (CM MET)$_2$ (CM$_2$ HIS)$_3$ cytochrome c HIS 18 is dicarboxymethylated which prevents its coordination with the heme iron. Since HIS 26 is on the surface and is only involved in an hydrogen bond with PRO 44 (24), it is unlikely that this residue plays a critical role in maintaining the native conformation. Upon examination of the amino acid sequence of cytochromes c of several species, this residue is found to be substituted only in one specie, the substitution being a conservative one (51).

Expected $\Delta\varepsilon_{395}$ for Ligand Coordination

When the acid unfolded molecule is refolded by neutralization a total $\Delta\varepsilon$ of 11.5×10^4 was observed at 395 nm (Table 1). The contribution of ligand coordination and crevice formation to this extinction change can be estimated from the spectral studies with model compounds. Using the $\Delta\varepsilon$ values observed for coordination of ligands in the fifth and sixth postion an average $\Delta\varepsilon$ value of $9.3 \pm 0.7 \times 10^4$ is obtained (Table 1). This value is approximately 80% of the total $\Delta\varepsilon$.

The contribution of the absorbance changes due to

changes in the heme environment to the total $\Delta\varepsilon$ can be calculated from the solvent perturbation difference spectra measurements. A value of 0.9×10^4 was estimated by multiplying the observed $\Delta\varepsilon$ at 395 nm obtained with HP 14-21 in the solvent perturbation experiment by 6 and 0.67. This correction was made because a polypeptide is about a 6-fold better perturbant than 20% ethylene glycol (54) and because the heme moiety in native cytochrome c is only 2/3 buried (24,28). A value of 0.4×10^4 was calculated in the same way using the $\Delta\varepsilon$ obtained with HP 11-21. In this fashion, the $\Delta\varepsilon$ at 395 nm obtained with model compounds give a total value of $10.2 \pm 0.7 \times 10^4$, a value that is somewhat lower than the one observed in the refolding transition. In attempting to resolve the total $\Delta\varepsilon$ at 395 nm, it must be recognized that a change in ligand orientation, a change in the ligand-iron bond length, or a change in the angle of the ligand-iron bond can all change the electronic properties of the heme iron (55) rendering the use of model compounds only a first approximation at best.

A similar calculation of the $\Delta\varepsilon$ at 620 nm was not possible since due to the low extinction at this wavelength, high concentration of hemopeptides have to be used, which increases the fraction of polymerized molecules.

Overall Rate of Refolding

The transition of cytochrome c from the unfolded state to the folded native conformation can occur very rapidly. As shown by kinetic measurements at 395 and 620 nm at pH 7 and 25°, most of the absorbance change, and probably the main folding, takes place with a half time as low as 2 msec, while the absorbance changes leading to the state characteristic of the native state occur with a half time of about 3 sec. These values are within the range of the times estimated for the *in vivo* synthesis of polypeptides and appearance of enzymatic activity. Using a rate of ribosomal travel of 1,000 nucleotides per min at 30° (56) a time of approximately 20 sec is estimated for synthesis of a molecule of the length of cytochrome c. The time necessary for the formation of the tertiary structure from the finished polypeptide has been estimated to be of the order of seconds to minutes (57,58). This indicates that the folding of polypeptide chains occurs very rapidly and that the active conformation is obtained shortly after synthesis. A slower folding process *in vivo* is unlikely since unfolded polypeptides are more suceptible to attack by proteases.

Presence of Stable Intermediates

In a transition of the type

$$U \rightleftharpoons N \qquad I$$

where U represents unfolded protein molecules and N represents folded native molecules, intermediate states between U and N must exist along the reaction pathway. Because of the cooperativeness of the folding process most of the intermediate conformations between U and N will have relatively low stability. If all intermediates are transient states, i.e. they are not present in significant amounts, the transition becomes a two-state transition and all but a small fraction of the molecules are either in the unfolded or native state. Consequently, first order kinetics will govern the entire transition. As exemplified in Figure 18, a first order plot of the absorbance changes accompanying the refolding of cytochrome c did not show such a behavior demonstrating the presence of a stable intermediate(s). Likewise, the absence of an isosbestic point in the spectra of the protein in the Soret and visible regions at intermediate pH values between pH 2 and 7 (Figures 3 and 4) also suggests that the refolding of the protein does not involve a simple equilibrium between unfolded and native molecules.

Possible Mechanism

In attempting to elucidate the mechanism of the refolding process, the number and nature of the intermediates is of importance. Analysis of the data obtained for the refolding of cytochrome c as in Figure 18, i.e. as parallel first order reactions, indicates the formation of at least two stable intermediates during the entire process. At this point it is of importance to emphasize that this method of analysis was chosen because of its simplicity and that other types of mechanism, e.g. sequential mechanism, are also consistent with the form and resolution of the kinetic data (19,59). When the refolding reaction was studied by following the change in absorbance at several wavelengths in a pH jump from pH 2 to pH 3 (Table 2), and the changes in absorbance at 395 nm in pH jumps from pH 2 to pH 3.2-7.2 (Table 4), three first order rate processes are obtained when the kinetic data is analyzed as in Figure 18. A simple mechanism consistent with the formation of two intermediates during the refolding process is shown by Eq. II

$$U \xrightarrow{k_1} X_1 \xrightarrow{k_2} X_2 \xrightarrow{k_3} N \qquad \text{II}$$

Since, under the conditions of the experiments, the equilibrium lies strongly on the side of the product, N, only the forward rates are considered. Eq. II represents

a mechanism in which the unfolded protein sequentially folds into intermediates X_1 and X_2, and then X_2 further folds to produce the final product N. This mechanism would be consistent with the form of the kinetic curves and its resolution into three first order processes.

As shown by equilibrium measurements (Table 1), most of the absorbance change accompanying the refolding of the protein is associated with the coordination of ligands to the heme iron. Likewise, the kinetic measurements at 395 nm (Tables 2 and 4) indicate that most of the absorbance change is associated with the two fastest refolding reactions. The $\Delta\varepsilon$ values associated with k_1 and k_2 are comparable with the observed values for coordination of a ligand in the fifth and sixth position respectively. The $\Delta\varepsilon$ value associated with k_3 is comparable with the value calculated for crevice formation. Consequently, in Eq. II, X_1 and X_2 would represent intermediates with one and two intrinsic ligands coordinated to the heme iron, respectively. The faster reaction with rate constant k_1 probably represents the coordination of HIS 18, since a large conformational change is not required to bring it in contact with the coordination site. The collapse of the unfolded structure probably occurs concurrent with the coordination of HIS 18. The slower absorbance change associated with k_2 probably reflects the coordination

of MET 80 that would require a substantial conformational change together with the specific residue interactions and interactions between the side chains and the heme. The absorbance change associated with k_3 probably reflects both the rate of conformational change that accompanies the heme burial after coordination has occurred and spectral subtle changes due to ligand rearrangements.

The events described above are reflected in all the wavelengths studied (Table 2). The rate assigned to coordination of HIS 18 does not seem to be reflected at 290.5 nm. If absorbance changes in the ultraviolet region are mainly due to the burial of the heme moiety and of the aromatic amino acid side chains, little or no absorbance change at 290.5 nm would be expected during coordination of HIS 18 because the main folding would not occur at this stage. The different values of k_1 at different visible wavelengths, if all reflect coordination of HIS 18, is not understood. Since a change in the spin state of the heme iron, reflected in a decrease in absorbance at 620 nm, also causes a change in the Soret maximum, changes at 410 and 395 nm are expected to be simulataneous with changes at 620 nm. However, if changes in the positions of the ligands relative to the heme moiety are mainly reflected in the Soret region, slower absorbance

changes in this region would be expected during folding of the polypeptide chain after ligand coordination. The values of k_2 and k_3 did not show a great difference at the different wavelengths studied.

Since the 695 mn band is absent when MET 80 is modified, the absorbance at this wavelength is probably a reflection of a small conformational change. However, absorbance changes at 695 nm were detected at all stages during the refolding process. When the kinetics of recovery of the 695 nm absorbance are studied in the alkaline region, only one rate process is observed (60,61). The first order rate constant was found to be 0.076 sec^{-1} for a pH jump from pH 10 to 7.0 (61), and 0.048 sec^{-1} for a pH jump from pH 10 to 7.6 (60). These values are much smaller than the values of k_1 and k_2 calculated at 695 nm for a pH jump from pH 2 to 3 (Table 2), but comparable with the value of k_3. Since the conformational change that occurs in the pH range 10-7 is very small (62), it is possible that the events associated with k_3 in the refolding of the protein are of the same nature as those occurring between pH 10 and 7. Since the absorbance at 695 nm is a measure of the 695 nm band per se and of the background absorbance, it is possible that k_1 and k_2 are a reflection of the latter.

The study of the effect of pH on the rate constants

obtained at 620 nm during the refolding of the protein suggests that the refolding mechanism is more complex than the one shown by Eq. II. When the final pH of the refolding reaction studied at 620 nm is higher than pH 4, after the initial decrease in absorbance a transition characterized by an increase in absorbance was observed. This suggests that the refolding at this pH proceeded by a different mechanism than at lower pH values. It is possible that upon coordination of HIS 18, two intrinsic ligands, one of them MET 80, compete for the sixth coordination position. Below pH 4 one ligand dominates the competition while above pH 4, the coordination of a ligand other than MET 80 forms a detectable intermediate. The cleavage of the coordinate covalent of the "wrong" ligand with the heme iron followed by formation of the coordinate covalent bond with MET 80 would account for the slow rates of increase (k_{-2}) and decrease (k_3) of absorbance at 620 nm. Alternatively, upon refolding of the protein the absorbance decrease at 620 nm is accompanied by a large increase in absorbance of a band at 530 nm with a shoulder around 560 nm (see Figure 2), and it is possible that this absorbance increase is responsible for the increase in absorbance detected at 620 nm.

The kinetic experiments done with (CM MET)$_2$ cytochrome c also suggest that the refolding reaction takes place

with the formation and cleavage of a coordinate covalent bond between the heme iron and a ligand that is not found to be coordinated in the final product. In a pH jump corresponding to the entire refolding transition (see Figure 22) the $\Delta\varepsilon$ associated with the fast initial decrease in absorbance has a value comparable to the one expected for coordination of two ligands (see Tables 2 and 4). The $\Delta\varepsilon$ of the subsequent increase in absorbance is somewhat smaller than that expected for the cleavage of one coordinate covalent bond. This diminution probably reflects the kinetic overlap of the dissociation of a "wrong" ligand and the coordination of the "proper" ligand. That this could be the case is suggested by the subsequent decrease in absorbance with a $\Delta\varepsilon$ expected for coordination of one ligand. The process associated with k_3 was not observed with this carboxymethylated derivative. When only a fraction of the complete transition was studied by lowering the final pH from 7.2 to 2.8 (see Figure 9), a transition involving an increase in absorbance was not observed, and the decrease in absorbance was resolved into two first order processes (Table 4). This is probably a reflection of the coordination of HIS 18 and a "wrong" ligand. In this derivative the events leading to the formation of the folded structure are more separated in the time scale compared to the unmodified protein because

of the modification of MET 80. The CM MET group in $(CM\ MET)_2$ cytochrome c would coordinate at a slower rate than the MET group in the unmodified protein because of the positive charge on the sulfur.

Little can be said about the identity of the "wrong" ligand that is proposed to coordinate in the intermediate formed during the initial stages of the refolding process. It could be that this residue is located close to the heme iron in the unfolded conformation, or that it is on the same side of the heme as MET 80 after substantial folding has occurred. Likely candidates are residues that are not charged below pH 7 and that form a low spin complex with the heme iron, such as histidine and tryptophan. Since two histidyl residues, HIS 18 and probably HIS 33, are found to be coordinated to the heme iron in a hemopeptide containing the first 65 residues of the protein (48), it is possible that a similar complex is formed during the initial stages of folding. However, such a situation would require a substantial rearrangement of the polypeptide chain in order to form the final product since all histidyl residues are located on one side of the heme in the native protein.

The study of the effect of pH on the refolding reactions was complicated by the marked increase in the observed rates. The pH profiles for the rate constants

k_1 and k_2 at 395 and 620 nm suggest that a group(s) with an apparent pK around 4 plays an important role in the refolding process. Whether this pK reflects the dissociation of a single group, such as an imidazole, or the dissociation of several groups, such as many of the γ and δ carboxyl groups, cannot be ascertained.

When the absorbance change at 395 nm are studied at 25°, the $\Delta\varepsilon$ value associated with k_1 is larger than that measured at 5°, while the $\Delta\varepsilon$ value associated with k_2 is smaller at 25° than at 5° (Table 4). Since the $\Delta\varepsilon$ value associated with k_1 at 25° is comparable with the one obtained upon coordination of two ligands, it is likely that k_1 represents the rate of coordination of two ligands at this temperature. The $\Delta\varepsilon$ value associated with k_2, somewhat smaller than the value expected for coordination of two ligands, probably represents the kinetic overlap of the cleavage of the coordinate covalent bond between the heme iron and the "wrong" ligand and the formation of the coordinate covalent bond between MET 80 and the heme iron, transitions that are accompanied by an increase and a decrease in absorbance at 395 nm, respectively.

The unfolding transition studied at 395 nm was also analyzed as being due to three first order reactions, but the values of the rate constants were considerably smaller

than the ones calculated for the refolding reactions. At this time little can be said about the events leading to the appearance of the unfolded protein, but since most of the absorbance change is associated with k_2 (Table 3), the rupture of the coordinate covalent bonds probably occurs at this stage.

The kinetic data obtained with model hemopeptides (Table 4) did not contribute to a better understanding of the kinetic system under study. Probably the tendency of these peptides to aggregate was reflected in the several rate processes observed. When a pH jump corresponding to the first step in the spectrophotometric titration of HP 11-21, coordination of HIS 18, was studied in the stopped-flow, the observed rate was slower than the one assigned to the same process in cytochrome c. The conformational change of the polypeptide chain of cytochrome c would facilitate the formation of the HIS-iron bond and this would be reflected in a faster coordination rate. When a pH jump corresponding to the whole spectral transition was studied, k_1 was too fast to be estimated and several other transitions were observed.

The refolding experiments done by dilution of the protein in urea or GuHCl also showed the presence of stable intermediates. However, the interpretation of the kinetic analyses is complicated by the fact that probably

two intrinsic ligands are coordinated to the heme iron in the presence of high concentrations of denaturing agent and because the state of the protein after dilution is not known. It is possible that after dilution of the denaturing agents the ligands are still the same, or they are the ones characteristic of the native protein. In both cases, the total observed $\Delta\varepsilon$ value should be the one expected for burial of the heme and aromatic residues. The $\Delta\varepsilon$ at 395 nm observed upon changing the concentration of urea from 0.8 to 8 M urea was found to be 1.5×10^4, a value that is lower than that expected for coordination of one ligand, either in the presence or absence of urea (Table 1), and somewhat higher than the expected value for heme burial. These observations together with the fact that the absorbance changes in the Soret and visible regions do not correlate with the viscosity changes (25a) indicate that absorbance in these regions is not an appropriate parameter to use when the conformational changes of cytochrome c are studied using urea or GuHCl as denaturants.

The kinetics of the absorbance changes observed at 400 nm upon denaturation and renaturation of cytochrome c in GuHCl at pH 6.5 and 25° have been used to elucidate the nature of the refolding process (63). These studies are found to be consistent with a mechanism where two

intermediates are formed during folding of the protein, one of which is not in the direct pathway between the native and denatured states. This conclusion, although it is consistent with the kinetic results obtained in this work, might be erroneous since a structureless polypeptide was assumed to be present at high concentrations of denaturing agent.

Folding in vivo

The site of synthesis and the sequence of events leading to the appearance of cytochrome c in the cell is not a settled problem. Cytochrome c is present predominantly in the mitochondria, but this organelle is not the site of its synthesis (64,65). The endoplasmic reticulum is believed to be the site of synthesis of at least the polypeptide chain of cytochrome c (64,65). The site and mechanism of attachment of the prosthetic group is not known with certainty. It has been suggested that it occurs before transfer of the completed protein to the mitochondria (65), by a combination of the apoprotein with protoporphyrinogen and subsequent insertion of iron (66), and by attachment of heme to the polypeptide chain as it comes off the ribosome (67). If the holoprotein is made by a combination of the apoprotein with heme, a mechanism similar to the one proposed in this work could prevail. If the heme is attached during synthesis of the polypeptide,

an intermediate where HIS 18 and HIS 26 or 33 are coordinated to the heme, would be present at least until MET is attached in position 65. This complex formation between histidine residues and heme is not unexpected. This residue has much higher affinity for heme than most amino acid residues (35), and two histidine residues have been found to be coordinated to the heme group in hemopeptides 1-26 and 1-29 (68) and 1-65 (48). Also, the spectral studies of this work done with the whole protein and model peptides suggest that two histidine residues are heme ligands even in the presence of high concentrations of denaturing agents, giving an idea of the strength of the interaction.

Refolding of Other Proteins

Kinetic studies of the refolding of a few proteins indicate that the process occurs with the formation of stable intermediates as it does with cytochrome c. Reconstitution studies of cytochrome b_{562} (21) followed by absorbance changes has been interpreted to involve a minimum of four reaction steps. At pH 7.0, the first step occurs in less than 5 msec, and the subsequent steps have first order rate constants of 20.9, 2.64, and 0.098 sec^{-1}. The kinetics of refolding of Staphylococcal nuclease (22), measured by fluorescence changes, is shown to be a fast process and is described as a sequence of two first order processes with rate constants of 12.1 and

1.9 sec^{-1}. Combination studies of porphyrins and globin (20) have been shown to occur in two stages, the first a very rapid reaction of the order of 5×10^8 M^{-1} sec^{-1} interpreted as binding of heme to globin, and a reaction with a rate constant of 40-370 sec^{-1} that was tentatively identified with the folding of the polypeptide chain around the heme group once binding occurs. The iron-histidine bond was suggested to occur in the first step since most of the spectrophotometric change was associated with the rapid phase. However, when the regeneration of acid denatured hemoglobin is studied (69), the 370 nm band, characteristic of heme, is found to disappear in 2 min or less, while the Soret band may take hours to reappear completely. The spectral changes accompanying the regeneration of acid denatured horse and human hemoglobin (69,70) have been interpreted in terms of the existence of transient intermediates. The refolding of β-lactoglobulin has been found to occur in three distinct stages (23) but the value of the rate constants associated with each process have not been reported. The renaturation rates of Ascaris collagen, at pH 6.5, studied by optical rotation, have been interpreted in terms of three first order processes with half times of about 0.20, 2.5 and 11 min (see reference 22).

The refolding rates observed with cytochrome c

appear to be faster than most of the renaturation rates of these proteins. It is interesting that most of the refolding processes so far studied have been observed to take place with the formation of stable intermediates. The first step can be ascribed to be due to a transition from an expanded to a more globular conformation, with or without the formation of a structure resembling the final equilibrium state of the protein. The subsequent slower processes are probably due to more subtle conformational changes leading to the structure characteristic of the native state.

MATERIALS AND METHODS

Materials

A. Proteins

Horse heart ferricytochrome c types III and VI were obtained from Sigma Chemical Co. Trypsin, chymotrypsin, and pepsin, were obtained from Worthington Biochemical Corp.

B. Chromatographic Materials

Sephadex G-50, fine grade, was obtained from Pharmacia. Whatman cellulose phosphate P 70 was obtained from W. and R. Balston Itd., England. Amberlite CG-50 was obtained from Mallinckrodt Chemical Works.

C. Reagents

Guanidine hydrochloride was obtained from Eastman Kodak Co. and was recrystallized from methanol. Urea, ultra pure grade, was obtained from Mann Research Laboratories Inc. Bromoacetic acid was obtained from Eastman Kodak Co. and recrystallized from petroleum ether.

Methods

A. Protein Concentration

The concentration of solutions of cytochrome c was determined spectrophotometrically using the extinction coefficients of Margoliash and Frohwirt (71). The protein solution was diluted into phosphate buffer, pH 7.0, and the absorbance at 410 nm was determined.

The concentration of chemically modified cytochrome c and hemopeptides was determined by amino acid analysis. The number of μ moles of amino acids stable to acid hydrolysis, such as aspartic acid, glutamic acid, and alanine, were divided by the number of residues of each amino acid present in each derivative. These values were averaged to obtain the concentration of the sample. This value of concentration, together with absorbance values, permited the calculation of the extinction coefficient for the derivatives and peptides.

B. Amino Acid Analyses

Protein and peptide solutions were diluted with an equal volume of 12 N HCl and placed in an hydrolysis tube. The samples were flushed with nitrogen, frozen in dry ice-acetone and then evacuated. The samples were flushed with nitrogen and evacuated three times. After the final evacuation, the hydrolysis tubes were sealed and placed

in a 110 ± 1° C oven for 24 hours. Samples were evaporated to dryness at reduced pressure and then dissolved in 1 ml of 0.2 M citrate buffer, pH 2.2. Aliquots of this solution were applied to the analytical columns of a Spinco Model 120 C amino acid analyzer. Acidic and neutral amino acids were resolved on the long column (54 cm) with 0.2 M citrate buffer, pH 3.15. The buffer change to the pH 4.25 buffer was set at 69 minutes. Basic amino acids were resolved on the short column (9 cm) with 0.35 M citrate buffer, pH 5.28. The ninhydrin and buffer solutions were prepared as described in the Beckman 120 C operation manual. The ninhydrin solution was standardized with Beckman type 1 calibration mixture. All analyses were performed at 56°.

C. Chemical Modifications

1. Methionyl Residues

After reaction of cytochrome c with 0.2 M bromoacetate for 48 hours in 0.1 M acetate at pH 2, only the two methionyl residues of the protein are carboxymethylated (39). Cytochrome c type III, 200 mg, was dissolved in 40 ml of a 0.2 M citrate, 0.2 M bromoacetic acid, pH 3 solution at room temperature during 6 and 28 hours. After the indicated times, an aliquot of the reaction mixture was neutralized and diluted about 25-fold with water and

applied to a 1.5 x 3 cm column of Amberlite CG-50 equilibrated with 0.02 M phosphate pH 7.0. Elution was carried out with 0.3 M KCl in the same buffer. Most of the heme colored protein was eluted at 0.3 M KCl. The protein solution was then exhaustively dialyzed against water, lyophilized and stored at -15°. Amino acid analyses of the material after 6 and 28 hours of reaction with bromoacetate gave 3.2 and 3.1 residues of histine assuming the presence of 2.0 arginine residues per molecule of modified protein. Thus, none of the three histidine residues present in the protein was modified under the conditions of the reactions. Because of the reversion of carboxymethyl methionyl residues to methionine during acid hydrolysis, an indirect procedure was employed to determine the extent of modification of the methionyl residues. As described by Neuman et al. (72), methionyl but not carboxymethyl methionyl residues are oxidized by performic acid to acid-stable methionyl sulfone residues prior to acid hydrolysis. In this way the methionine sulfone content of the sample represents the number of methionine residues that did not react to form the carboxymethylated derivative. The modified protein was subjected to performic acid oxidation as described by Hirs (73). An aliquot of the modified protein solution was dialyzed against water, lyophilized and dissolved in 1 ml of formic acid followed by 2 ml of performic acid. After

reaction for 2.5 hours at 2°, 100 ml of iced water was added to the reaction mixture, and the sample was then frozen and lyophilized. Lyophilization was repeated once more after adding 25 ml of water to the dry protein. The residue was dissolved in 6 N HCl for amino acid analysis. The methionine sulfone content was calculated from the ratio of sulfone and aspartic acid, assuming that the ninhydrin color value was the same for both amino acids and that the moles of aspartic acid represented eight aspartate residues per molecule of the modified protein. After 6 hours of reaction with bromoacetate, 0.2 residues of methionine sulfone were found. The material obtained after 28 hours of reaction with bromoacetate was not oxidized with performic acid. However, no methionine was found in the amino acid analysis. A molar extinction of 127×10^3 at 406 nm and at pH 8 was determined for this derivative. This derivative is referred as $(CM\ MET)_2$ cytochrome c.

2. Methionyl and Histidyl Residues

Only one of the three histidyl and one of the two methionyl residues of cytochrome c is carboxymethylated by reaction with 0.2 M bromoacetate at pH 7.0 for 3 days, as described by Stellwagen (39). In the presence of 8 M urea, all five residues can be carboxymethylated after five days of reaction (42). This carboxymethylated

derivative was prepared as follows. Cytochrome c type III was made 10 mg/ml by dissolving it in 10 mls of a solution of 0.1 M phosphate pH 7.0 and 9 M urea. After 7 days at room temperature the protein was dialyzed against water for 3 days (4 x 2 lts), lyophilized and stored dry at -15°. This derivative is referred as $(CM\ MET)_2$ $(CM_2\ HIS)_3$ cytochrome c. Free histidine was not present in the hydrolyzate of this derivative but 2.6 residues of the 1,3 dicarboxymethyl derivative of this amino acid were found. The 1,3 dicarboxymethyl histidine content was calculated from the ratio of this derivative and aspartic acid, assuming that the content of aspartic acid represented eight aspartate residues per molecule of modified protein. A color value of 0.96 relative to serine was used to quantitate the 1,3 dicarboxymethylated derivative as reported by Crestfield et al. (74). No methionine sulfone was detected upon oxidation of this derivative with performic acid, indicating the conversion of methionyl to carboxymethyl methionyl residues. Besides modification of histidyl and methionyl residues, the modification of lysyl residues also seemed to occur under the conditions of the reaction. In the amino acid analysis a peak near the elution position of methionine, and identified as ε-monocarboxymethyl lysine (75), was observed, and corresponded to 2.3 residues. A molar extinction of 97×10^3 at 390 nm and pH 9 was determined

for this derivative.

The hemopeptide 11-21 (see Preparation of Peptides) was carboxymethylated in the same way as described above but the reaction was stopped after 5 days. After that period of time 0.2 moles of histidine and 0.4 mole of dicarboxymethyl histidine were detected by amino acid analysis assuming that the content of aspartic acid represented two aspartate residues per molecule of peptide.

D. Preparation of Hemopeptides

Hemopeptides containing residues 11 to 21 (HP 11-21) and 14 to 21 (HP 14-21) were prepared as described in the literature (76). HP 11-21 was obtained by treatment of cytochrome c with pepsin and HP 14-21 by action of trypsin on HP 11-21. The amino acid composition of the peptides agreed very well with the theoretical values. A molar extinction of 95 x 10^3 was calculated for HP 14-21 at 398 nm pH 6.5 and 106 x 10^3 for HP 11-21 at 406 nm pH 10.

E. Viscosity Measurements

Viscosity measurements were made with an Ostwald viscometer with an outflow time of about 81 seconds for 4 ml of water. The experiments were carried out at 24.6° in a well stirred bath; temperature variations, as detected by a Beckman thermometer, did not exceed ± 0.01°. The samples used to study the effect of decreasing pH

(unfolding) and increasing pH (refolding) on the viscosity of cytochrome c were prepared as follows. For the unfolding transition, a solution of approximately 4 mg/ml of cytochrome c (type VI or III) in distilled water was adjusted to a certain pH and dialyzed overnight against water at the same pH. For the refolding transition after the dialysis step the protein solution was adjusted to pH 2.15 by addition of HCl and then adjusted to higher pH values by addition of NaOH. An interval of at least 30 min elapsed between measurements. A similar procedure was used for studying the effect of pH on the viscosity of chemically modified cytochromes. Before measuring the outflow times all solutions were filtered through 1.2 μ Millipore filters. An average of at least five determinations was used for the outflow time of each sample; the outflow times had a mean absolute deviation of ± 0.05 sec. After a series of readings were made on a solution the viscometer was carefully cleaned. The procedure adopted consisted in removal of the protein solution from the viscometer, rinsing it several times with distilled water, cleaning with hot trisodium phosphate solution, rinsing again with distilled water, and finally drying by flowing air through it. This treatment was found to restore the solvent outflow time. All values are reported as reduced viscosities in g per ml.

The reversibility and stability of the acid denatured protein was studied as follows. Cytochrome c was maintained during 14 hours in a 0.01 N HCl solution at room temperature. After this period of time the solution was diluted 1:2 (v/v) with 0.05 M phosphate buffer and adjusted to pH 7.0. Then it was dialyzed against 0.025 M phosphate pH 7.0 during 10 hours and its viscosity was measured. A protein solution not exposed to HCl served as control.

F. Spectral Measurements

The salt free lyophilized protein or peptide was dissolved in water or in a KCl solution of a desired concentration and then concentrated NaOH or HCl was added to adjust the pH to its initial value. The pH was measured with a Radiometer Model PHM 26 pH meter equipped with a combined glass-calomel electrode and with a ten fold scale expander. The equilibrium absorbance measurements were made with a Gilford spectrophotometer in a 1 cm cell. The temperature for the pH and absorbance measurements was room temperature (24 $\pm$ 1°). Spectra, difference spectra, and solvent perturbation difference spectra were obtained with a Cary model 14 recording spectrophotometer. The technique used in the solvent perturbation measurements is described in detail by Herskovits and Laskowski (77). Ethylene glycol at a concentration of 20% by volume was used as a perturbant

of the heme moiety. Stoppered tandem cylindrical quartz cells with a volume of 3 ml in each compartment were used. Compartment A of the reference cell contained 2.4 ml of peptide solution, and 0.6 ml of water; compartment B, 2.4 ml of solvent and 0.6 ml of ethylene glycol. Compartment A of the sample cell contained 2.4 ml of peptide solution, and 0.6 ml of ethylene glycol; compartment B, 2.4 ml of solvent and 0.6 ml of water.

G. Stopped-Flow Measurements

All kinetic measurements were made with a Durrum-Gibson stopped-flow spectrophotometer equipped with a Techtronix storage oscilloscope. The system operates as follows. The drive syringes are filled with the reagents to be mixed and, by means of a flow actuator, equal volumes of the two reactants (0.3 ml) are forced to mix in the mixing jet and flow through the cuvette. When the stop syringe is filled, the flow of solution is stopped and the trigger switch is actuated. This initiates the horizontal time base sweep for the oscilloscope display. Monochromatic light passes through the mixed solution in the cuvette (20 mm) during the reaction, and the resultant intensity light is projected into the photomultiplier tube. The photomultiplier output drives the vertical axes of the oscilloscope. The resultant display indicates transmitted light intensity vs. time starting at the same

time or shortly before flow of solution is stopped.

The following is a description of the different steps followed during operation of the stopped-flow apparatus in a refolding experiment. The oscilloscope and power supply were turned on at least one hour before initiation of measurements. Also, water was circulated through the mixing chamber at the desired temperature allowing enough time for equilibration to occur. For each experiment, three different air free solutions were made: 1) a cytochrome c solution in 0.01 N HCl of a concentration that varied depending on the wavelength used, 2) a NaOH solution of a concentration such that when mixed (1:1 v/v) with the cytochrome c solution the desired final pH was obtained and 3) a 0.01 N HCl solution. These solutions were made fresh each time, and the protein was not exposed to 0.01 N HCl less than 1 hr or more than 6 hr before each measurement. These solutions were used to fill the drive syringe, as recommended in the Durrum Operation Manual, being careful not to aerate them and to eliminate all air bubbles. One driving syringe was filled with the protein solution and the other was filled with the NaOH solution. After this was done, the oscilloscope graticulate was calibrated in the following way. With the oscilloscope vertical volts/div control at 0.1 volts/div and the oscilloscope (+) and (-) inputs at GND, the

vertical position control was adjusted to set the trace on the bottom horizontal line of the oscilloscope screen (0% T). Then, with the vertical (-) input switch at DC and the (+) at GND, several trial determinations were made, and by adjusting the kilovolts switch together with the voltage control on the photomultiplier power supply, the trace was moved to the top horizontal line of the oscilloscope screen (100% T). The 100% T value was always set with the reaction mixture in the cuvette and allowing enough time for the reaction to be completed. In this way the screen was calibrated to provide a 0 to 100% full scale display. If a more detailed display of the scale was desired (90 to 100% T for example) it was obtained by offsetting a portion of the full scale amplitude by means of the zero offset control and by setting the vertical volts per division selector to the proper value (10 mv for the 90 to 100% T range). The time constant settings were usually 0.1 or 1.0 msec and the slit 1.0 mm. After the data for one experiment was collected, the NaOH solution was replaced with the 0.01 N HCl solution and the T value obtained after mixing represented the value of the initial reaction mixture, that is, the value obtained after dilution without change in pH.

When unfolding experiments were performed, the protein in neutral solution was mixed with acid to give the desired

final pH values. When refolding experiments were done using urea or Gu HCl, the protein solution in 0.01 M phosphate pH 7.0 and in 8 M urea or 3.5 M Gu HCl was mixed with 0.01 M phosphate to give the final concentration of denaturing agent.

The oscilloscope traces were obtained over periods varying from 0.05 to 170 seconds depending on the conditions of the reaction. The earliest reading was obtained 5-15 msec after mixing. To facilitate the reading of the oscilloscope images, the oscilloscope images were traced onto tracing paper, and these copies were placed over graph paper on a ligh box. The oscilloscope readings, linear in transmittance, were converted to absorbances and used for kinetic analyses.

For construction of time dependent spectra, measurements at the same final pH were repeated at a number of different wavelengths; % T at particular times after mixing were then read from the traces, and absorption spectra for these times were constructed. The spectra illustrated were corrected for a light path length of 1 cm.

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