Horton, D., and Hutson, D. H. (1963), *Advan. Carbohydrate Chem. 18*, 123.
Hou, C. T., Umemura, Y., Nakamura, M., and Funahashi, S. (1967), *J. Biochem. (Tokyo) 62*, 389.
Ikawa, M., Morrow, J. W., and Harney, S. J. (1966), *J. Bacteriol. 92*, 812.
LePage, M. (1964), *J. Lipid Res. 5*, 587.
Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.
Phillips, D. D. (1954), *J. Am. Chem. Soc. 76*, 3598.
Plackett, P. (1967), *Biochemistry 6*, 2746.
Rapport, M. M., and Alonzo, N. (1955), *J. Biol. Chem. 217*, 193.
Rothblat, G. H., and Smith, P. F. (1961), *J. Bacteriol. 82*, 479.
Shaw, N., and Baddiley, J. (1968), *Nature 217*, 142.
Shaw, N., and Smith, P. F. (1967), *Bacteriol. Proc.*, 108.
Shaw, N., Smith, P. F., and Koostra, W. L. (1968), *Biochem. J.* 107.
Smith, P. F. (1963a), *J. Gen. Microbiol. 32*, 307.
Smith, P. F. (1963b), *in* Recent Progress in Microbiology, Gibbons, N. E., Ed., Toronto, University of Toronto, p 518.
Smith, P. F. (1967), *Ann. N. Y. Acad. Sci. 143*, 139.
Smith, P. F. (1968), *J. Am. Oil Chemists' Soc.* (in press).
Smith, P. F., and Koostra, W. L. (1967), *J. Bacteriol. 93*, 1853.
Sweeley, C. C., Bentley, R., Makita, M., and Walls, W. W. (1963), *J. Am. Chem. Soc. 85*, 2497.
Vilkas, E., Adam, A., and Senn, M. (1968), *Chem. Phys. Lipids 2*, 11.
Wells, M. A., and Dittmer, J. C. (1963), *Biochemistry 2*, 1259.
Welsh, K., Shaw, N., and Baddiley, J. (1968), *Biochem. J. 107*, 313.
Whistler, R. L. (1945), *Advan. Carbohydrate Chem. 1*, 279.

Hydrogen–Deuterium Exchange of Cytochrome *c*. I. Effect of Oxidation State*

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ABSTRACT: The hydrogen–deuterium exchange of oxidized and reduced horse heart cytochrome *c* has been examined by infrared spectrophotometry. At pD 7.4, about 59% of the peptide hydrogens of ferrocytochrome exchange rapidly, *i.e.*, within 5 min, 20% slowly, and 21% are unexchanged after 24 hr. Comparable values for ferricytochrome are 68, 21, and 11%. The constant difference of 9–10% is in accord with previous observations that the oxidized and reduced cytochromes differ in conformation and that the reduced protein is the more compact (Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113). Reversal of the redox condition of either cytochrome at the *initiation* of exchange instantly and completely alters the kinetic exchange curve to that typical of the new oxidation state. In contrast, oxidation of ferrocytochrome (or reduction of ferricytochrome) at intervals *after* initiation of exchange accelerates (or retards) the exchange reaction so as to *gradually* approach that characteristic of the new oxidation state. In this manner, it becomes apparent that the number of peptide hydrogens affected by the structural alteration is considerably larger than indicated from the 10% displacement of individual kinetic curves. This suggests that the oxidoreduction-induced change in conformation involves an appreciable portion of the cytochrome molecule.

Oxidation–reduction of the heme iron atom induces remarkable changes in the physicochemical properties of cytochrome *c*. Ferrocytochrome has greater thermal stability (Butt and Keilin, 1962) and resistance to proteolytic digestion (Nozaki *et al.*, 1957, 1958) than does ferricytochrome, and the two differ in antigenic characteristics (Margoliash *et al.*, 1967), in chemical reactivity of amino acid side chains such as methionine (Matsubara *et al.*, 1965) and tyrosine (Ulmer, 1966), and in the interaction of the heme group with ligands such as cyanide and azide (Theorell and Åkesson, 1941; Horecker and Kornberg, 1946; Horecker and Stannard, 1948). These, and related observations, have generally been interpreted to indicate that oxidized and reduced cytochrome *c* differ in conformation.

The nature of this conformational variation has been investigated recently by means of spectropolarimetry

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(Ulmer, 1965; Urry and Doty, 1965; Myer and Harbury, 1965). Oxidoreduction of cytochrome *c* induces marked changes in the asymmetry of the heme chromophores; however, the two redox forms show little difference in the magnitude of their *intrinsic* Cotton effects,[1] ordinarily taken as a reliable index of significant conformational alteration. Hence, it appeared useful to attempt to gauge the relationship of oxidation state to conformation by an alternative procedure.

Hydrogen–deuterium (H–D) exchange has been suggested as an exquisitely sensitive method for study of protein conformation since it measures the "reactivity" of groups positioned *throughout the entire molecule*, *e.g.*, all the peptide groups (Hvidt and Nielsen, 1966). Either localized or more general structural rearrangements might alter the exchange characteristics of certain peptide hydrogens and the number affected should bear some relation to the degree of conformational variation. Therefore, we have selected H–D exchange as an approach to further evaluation of the postulated structural difference between oxidized and reduced cytochrome *c*.

The results reveal a substantial difference in the kinetic exchange curves of the two forms and indicate that the structure of the reduced is significantly more compact than that of the oxidized cytochrome. Uniquely, cytochrome structure can be modulated by oxidation or reduction *during the exchange reaction* causing acceleration or retardation of the course of exchange. In this manner, it is shown that the redox-induced structural alteration affects the kinetic characteristics of an even larger number of peptide hydrogens than is apparent from the unmodulated kinetic curves.

The effect of pH on the exchange characteristics of the oxidized and reduced protein is discussed in an accompanying paper (Kägi and Ulmer, 1968).

Preliminary accounts of a portion of this work have been reported (Ulmer and Kägi, 1967).

Materials and Methods

Horse heart cytochrome *c* (type III) was purchased from Sigma Chemical Co. Deuterium oxide was obtained both from Bio-Rad (99.84% pure) and Volk (99.6% pure) laboratories. All D_2O-containing solutions were kept in sealed Pyrex flasks to prevent exchange with atmospheric water.

Preparation of Samples. Oxidized cytochrome *c* was prepared by dissolving up to 150 mg/ml of protein in 0.01 M sodium phosphate (pH 7.0) containing 0.05 M sodium ferricyanide. Reduced cytochrome was prepared by dissolving a similar concentration of protein in 0.01 M sodium phosphate–0.05 M ascorbate, freshly neutralized to pH 7.0 with NaOH. Ferricyanide and ascorbate were removed by passing the protein solutions over a column (1 × 30 cm) of Bio-Gel-P2 (Bio-Rad Laboratories), equilibrated with 0.01 M sodium phosphate (pH 7.0). The solutions were adjusted to a final concentration of 4.9×10^{-3} M as determined spectrophotometrically at 550.3 mμ, on samples reduced with excess sodium dithionite, in 0.1 M sodium phosphate (pH 6.8), employing a millimolar extinction coefficient of 27.7 (Margoliash and Frohwirt, 1959).

From 200- to 2000-μl volumes of either oxidized or reduced cytochrome, prepared as above, were pipetted into individual microvessels (Buch and Holm Co., Copenhagen), frozen at −60°, and placed over P_2O_5 in a vacuum desiccator which was then evacuated with an oil pump (Hvidt *et al.*, 1960). After 20-hr lyophilization at room temperature the vacuum was released and the microvessels were sealed with greased caps to prevent uptake of water vapor during storage, at 4°, for 24–48 hr prior to the exchange experiments. Representative lyophilized protein samples were examined spectrophotometrically, in the presence and absence of excess ferricyanide or ascorbate, to assure that oxidation (reduction) remained complete prior to initiation of deuterium exchange. Blank samples of 0.01 M sodium phosphate buffer (pH 7.0) were prepared in an identical fashion.

Buffered solutions of D_2O were prepared by lyophilizing measured portions of aqueous buffer of the desired concentration and pH and redissolving the salts in the same volume of D_2O. Other solutes, *i.e.*, potassium ferricyanide, sodium ascorbate, and sodium dodecyl sulfate, were lyophilized separately from aqueous solutions and dissolved in buffered D_2O prior to the exchange experiment.

Deuteration of Cytochrome c. Rates of hydrogen–deuterium exchange were measured by means of infrared spectroscopy (Blout *et al.*, 1961) employing a Perkin-Elmer Model 521 double-beam grating infrared spectrometer operated in the normal mode.[2] Calcium fluoride cells of 0.05-mm path length were employed.

Exchange was initiated by rapidly dissolving individual samples of dry protein (12.2 mg) in 200-μl volumes of buffered D_2O, selectively adjusted to the appropriate pH, or in buffered D_2O of the appropriate pH containing up to five times molar excess of either ascorbate or ferricyanide. The starting time for each experiment was taken as the instant at which solvent was added to the microvessel since the time required for solubilization was variable (30–90 sec). Upon dissolution, the sample was immediately transferred to the calcium fluoride cells, the first measurement ordinarily being recorded in from 3 to 5 min after adding solvent. Blank solutions were prepared by adding identical volumes of D_2O to microvessels containing all constituents except protein.

The rate of hydrogen–deuterium exchange was determined by repetitive scanning of the spectral range 1750–1350 cm^{-1}. The times indicated in the text and figures refer to the moment at which the maximum of

[1] Terminology is in accord with the suggestion of Blout (1964), where *intrinsic* Cotton effects are those which appear to arise from the primary and secondary structure of proteins, *e.g.*, through conformational orientation of peptide bonds, as opposed to Cotton effects generated by prosthetic groups, bound metal atoms, or dyes which are designated *extrinsic*.

[2] The authors are grateful to Dr. E. R. Blout for the use of the infrared spectrophotometer and related facilities.

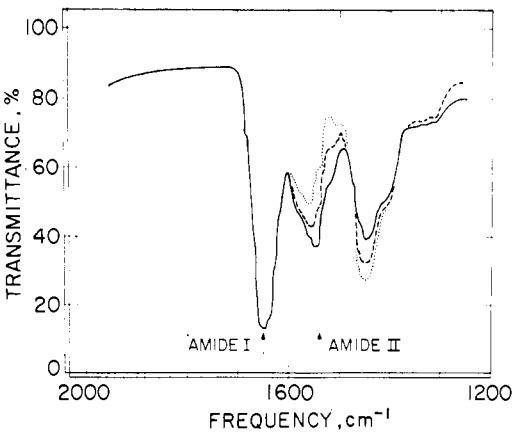

FIGURE 1: Infrared spectra of ferrocytochrome *c* in deuterium oxide. As hydrogen is exchanged for deuterium, the absorption of the amide II band, at 1540 cm^{-1}, decreases while the amide I band, at 1650 cm^{-1}, remains constant. In addition, deuteration of peptide groups and DHO formation during the exchange reaction generate a new absorption band at 1450 cm^{-1}. The denatured protein, heated to 45° in the presence of 0.2% sodium dodecyl sulfate, demonstrates maximal deuteration. Spectra were obtained by dissolving 12.2 mg of protein in 200 μl of D_2O buffered to pD 7.4 with 0.11 M sodium phosphate and measuring transmittance in 0.05-mm calcium fluoride cells against appropriate solvent blanks (see Methods). (——) 3.5 min; (- - - -) 24 hr; (· · · · ·) denatured.

the NH (amide II) absorption band (1540 cm^{-1}) was passed. The decrease in absorption of this band was followed as a function of time and, with each scan, the absorption of the amide I band (1650 cm^{-1}) was redetermined. Scan speeds of about 1 cm^{-1}/sec were employed in the region of the amide I and amide II peaks, since faster scanning speeds appeared to introduce errors in estimation of band intensities. All measurements were carried out at a constant room temperature (20 ± 1°), although the temperature of the sample increased somewhat while the cells were left in the beam of radiation. Because of the acceleration of exchange by heat, separate aliquots of protein were employed for successive points in the evaluation of kinetic exchange curves.

In those experiments where the redox state of the protein was altered *during the course of the reaction*, the cytochrome was initially dissolved in buffered D_2O and, subsequently, aliquots were removed and added to previously measured, neutralized, and lyophilized portions of potassium ferricyanide or sodium ascorbate (see Results). In these instances, the oxidation state of the protein was redetermined spectrophotometrically, and the pH of the solutions was verified, following completion of the experiments.

Blank measurements were made with each determination. The transmittancy of these solutions, like that of the corresponding cytochrome samples, was determined against a reference cell containing D_2O only.

The infrared spectra were recorded on a linear transmittancy scale with full-scale deflection adjusted to 0.95 of the chart width. The absorbance of the sample (log $1/T_S$) was corrected for the absorbance of the corresponding "blank" solution (log $1/T_B$) at each frequency.[3]

Cytochrome absorbance in the visible spectral range was determined in a Cary Model 15 recording spectrophotometer at a temperature of 23°, employing 1-cm path-length cells. Potentiometric measurements were made with a Radiometer pH meter equipped with a general purpose glass electrode or with a Beckman microglass electrode. In D_2O solutions, pD values were determined according to the recommendations of Glasoe and Long (1960).

Calculations of Hydrogen–Deuterium Exchange. The change in concentration of peptide hydrogens corresponding to a given change in intensity of the NH absorption band was calculated in a manner analagous to that suggested by Blout *et al.* (1961). The ratio of the apparent absorbance of the amide II (1540 cm^{-1}) to that of the amide I band (1650 cm^{-1}) in the unexchanged cytochrome was evaluated from spectra of the lyophilized protein in potassium bromide pellets;[4] this ratio was found to be 0.72 when the samples were lyophilized from solutions of neutral pH. The ratio for the maximally deuterated protein, at neutral pH, is 0.18 as obtained both by heating the protein to 45° in the presence of 0.2% sodium dodecyl sulfate (which results in precipitation of a portion of the material) and by neutralizing cytochrome solutions after 24-hr exchange at extremes of pH (1.5 and 12.0). The ratio, $A_{\text{amide II}}/A_{\text{amide I}}$, of 0.18 for the completely deuterated protein is identical with that reported for heavy meromyosin (Willumsen, 1966), while the difference between this value and that for the unexchanged cytochrome, 0.72 − 0.18 = 0.54, is close to the value found previously for several other globular proteins (Blout *et al.*, 1961). From this relative absorption coefficient of the amide II band, and assuming the validity of Beer's law, the percentage of the total number of peptide hydrogens which corresponds to a given change in absorbance is obtained from the relationship: % of total NH = $\Delta((A_{\text{amide II}}/A_{\text{amide I}})/0.54) \times 100$.

Results

Infrared spectra of ferrocytochrome *c* in D_2O (pD 7.4) at different stages of exchange are shown in Figure 1. The principal absorption band, at 1650 cm^{-1}, designated amide I, corresponds to the C=O stretching frequency of the peptide bond (Schellman and Schellman, 1964). The adjacent band has two major components. One originates from ionized carboxyl groups

[3] Oxidized and reduced ascorbate differ in their absorption at both 1650 and 1540 cm^{-1}; therefore, the relative proportion of each oxidation state of ascorbate present in protein samples was calculated from a band at 1830 cm^{-1}, present only in the reduced ascorbate, and the appropriate corrections were made for ascorbate absorption at the amide II and amide I bands.

[4] The apparent molar extinction coefficient for the carbonyl band ($k'_{C=O}$) at the protein concentrations employed and in 0.1 M sodium phosphate (pH 7.0) was found to be 368 l. $mole^{-1}$ cm^{-1}. This compares closely to the value reported for lysozyme ($k'_{C=O}$ = 353) (Hvidt, 1963) but is larger than that for yeast alcohol dehydrogenase ($k'_{C=O}$ = 272) (Hvidt and Kägi, 1963).

TABLE I: Hydrogen–Deuterium Exchange of Cytochrome *c*. Effect of Reversing Redox Condition at Initiation of Exchange.

Redox Condn (before dissoln)	Reaction[a]	% Rapidly Exchanging Peptide Hydrogen[b]
Ferricytochrome	None	68
Ferricytochrome	Oxidation	68
Ferricytochrome	Reduction	59
Ferrocytochrome	None	59
Ferrocytochrome	Reduction	59
Ferrocytochrome	Oxidation	68

[a] Cytochromes were dissolved in 0.11 M sodium phosphate–D_2O (pD 7.4) to a final protein concentration of 4.9×10^{-3} M, in the absence (no reaction) or presence of a 2 × molar excess of potassium ferricyanide (for oxidation) or sodium ascorbate (for reduction). Infrared spectra were recorded immediately. [b] Calculated from the ratio of absorbancies, amide II/amide I (see Methods).

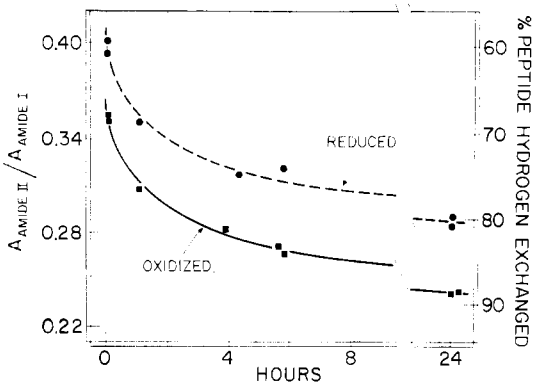

FIGURE 2: Time course of exchange of hydrogen for deuterium in reduced and oxidized cytochrome *c*, at pD 7.4. The ratio of absorbancies of the amide bands, amide II/amide I (left ordinate), and the corresponding per cent peptide hydrogen exchanged (right ordinate) are plotted against time. The oxidized protein appears to have exchanged nine to ten more hydrogens than the reduced throughout the entire period of observation. Conditions as in Figure 1 and Methods.

of the protein side chains (Ehrlich and Sutherland, 1954) and has a maximum at 1573 cm^{-1}. This band disappears at acid pH but at neutral and alkaline pH it strongly overlaps the amide II band of the peptide bond, which then appears as a shoulder at 1540 cm^{-1}. The amide II band arises from a coupled CN vibration and NH deformation frequency (Fraser and Price, 1952; Miyazawa *et al.*, 1958); its absorption decreases progressively in D_2O as hydrogen atoms are replaced by deuterium (Figure 1). Concomitantly, a new band appears at 1450 cm^{-1} arising both from deuterated peptide groups (Miyazawa *et al.*, 1958) and the DHO formed during the exchange reaction. These spectra are quite similar to those reported for other globular proteins (Blout *et al.*, 1961; Schellman and Schellman, 1964) and the positions of the bands are identical for the oxidized and reduced cytochromes. However, the *rate* at which the absorption of the amide II band decreases upon exposure to D_2O differs markedly for the two oxidation states.

Figure 2 compares the amide hydrogen–deuterium exchange of ferro- and ferricytochrome *c* over a 24-hr period. At pD 7.4 and 20°, about 59% of the peptide hydrogens of the reduced protein are exchanged very rapidly (within 5 min of dissolving the protein in D_2O). During the subsequent 24 hr, an additional 21% exchange more slowly, while about 20% remain unexchanged even at the end of this period. In contrast, in the oxidized protein, 68% of the amide hydrogens are exchanged rapidly. As with the ferrocytochrome, 21% exchange slowly while 11% remain unexchanged at 24 hr (Figure 2). Since in cytochrome *c* there are a total of 99 potentially exchangeable peptide amide hydrogens, at least 9 or 10 more hydrogens appear to be exchanged in the oxidized as compared with the reduced protein. This suggests that the macromolecular conformation of the two oxidation states differs and that the reduced protein is the more compact.

Under the conditions employed, exchange rates do not appear to be particularly sensitive to changes in ionic strength. It has been shown that bovine serum albumin undergoes a marked decrease in the rate of hydrogen exchange, at pH 7, when ionic strength is increased from 0.01 to 0.11 M (Benson *et al.*, 1964). However, at this pH, the exchange curves for cytochrome *c* were not influenced by varying the ionic strength over the same tenfold range. Both temperature and pH markedly affect the exchange characteristics of cytochrome *c*, as with most other proteins investigated to date (Hvidt and Nielsen, 1966), although only the influence of pH has been studied systematically in the present investigation (Kägi and Ulmer, 1968).

The redox condition of cytochrome *c* is reversible, instantaneously and completely. Since protein conformation appears to depend upon the redox state, this system provides an opportunity to assess the influence on exchangeable hydrogens of inducing a structural alteration at various intervals throughout the course of exchange.

The effect of reversal of the oxidation state of the heme iron atom at the initiation of exchange is shown in Table I. When samples of oxidized and reduced cytochrome *c* are dissolved in D_2O containing either ferricyanide or ascorbate in concentrations sufficient to reverse the redox state simultaneously with dissolution, instant and complete reversal of the exchange pattern is achieved. Oxidation of the ferroprotein results in a degree of exchange identical with that of the control oxidized protein (Table I). Conversely, reduction of the ferriprotein results in exchange characteristic of the control reduced cytochrome. In both instances, the

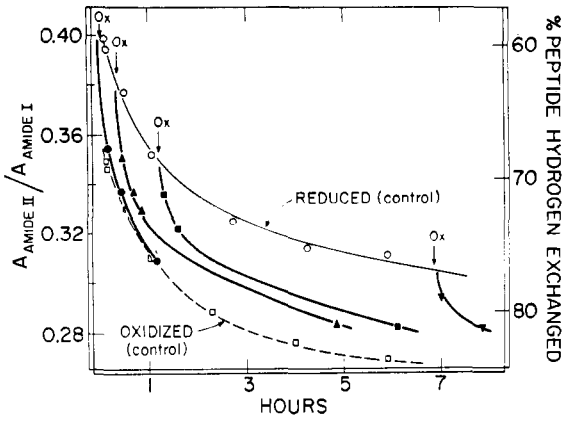

FIGURE 3: Effect on ferrocytochrome of oxidation at intervals during the time course of exchange of hydrogen for deuterium. The ratio of absorbancies of the amide bands, amide II/amide I (left ordinate), and the corresponding per cent peptide hydrogen exchanged (right ordinate) are plotted against time. The kinetic exchange curves of unmodulated reduced (○—○) and oxidized (□- - -□) cytochromes are shown as controls. Oxidation (Ox) of ferrocytochrome with potassium ferricyanide after 1.5-min exposure to deuterium rapidly accelerates exchange to that of the control oxidized protein (●—●). Oxidation of ferrocytochrome at later times, 21 min (▲—▲), 74 min (■—■), or 415 min (▼—▼), also accelerates exchange but there is a progressive decrease in the rate and extent to which the exchange curves approach that of the oxidized control. Conditions: protein concentration, 4.9×10^{-3} M, and potassium ferricyanide, 1×10^{-2} M, in 0.11 M sodium phosphate (pD 7.4). For experimental details, see Methods.

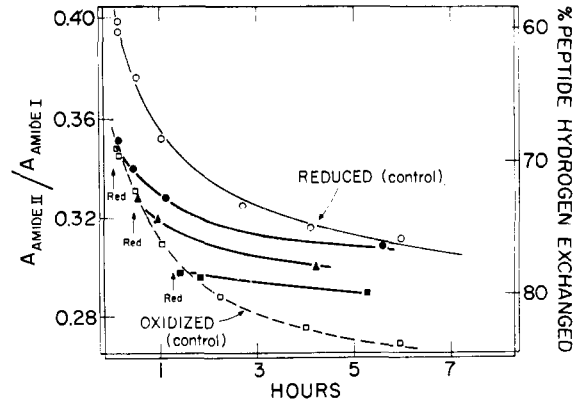

FIGURE 4: Effect on ferricytochrome of reduction at intervals during the time course of exchange of hydrogen for deuterium. The ratio of absorbancies of the amide bands, amide II/amide I (left ordinate), and the corresponding per cent peptide hydrogen exchanged (right ordinate) are plotted against time. The kinetic exchange curves of unmodulated reduced (○—○) and oxidized (□- - -□) cytochrome are shown as controls. Reduction (Red) of ferricytochrome with ascorbate after 1.5-min exposure to deuterium retards exchange and, after 6 hr, the extent of exchange approaches that of the reduced control (●—●). Reduction of ferricytochrome at later times, 28 min (▲—▲) or 78 min (■—■), also retards exchange although the value for the reduced control is no longer completely attained. Conditions: protein concentration, 4.9×10^{-3} M, and ascorbate, 1×10^{-2} M, in 0.11 M sodium phosphate (pD 7.4). For experimental details, see Methods.

exchange rates remain typical for the new redox form throughout the 24-hr period of observation.

The results are quite different, however, if the redox condition is reversed *after* initiation of the exchange reaction. Thus, Figure 3 shows the effect of oxidizing ferrocytochrome *c* at various times *after* dissolution in D_2O. The exchange curves of unaltered ferri- and ferrocytochrome are again measured as controls. If, after 1.5 min exposure to solvent, an aliquot of the ferrocytochrome solution is oxidized with ferricyanide, its exchange accelerates; within 5 minutes, total exchange nearly equals that of the control ferricytochrome at the same interval (Figure 3). Thereafter, the exchange curve of this aliquot parallels that of the ferricytochrome control. If oxidation is carried out at still later times during the exchange, *e.g.*, after 21, 74, or 415 min, acceleration is also observed but there is a progressive decrease in the rate and extent to which the exchange curve approaches that of the oxidized protein (Figure 3). Thus, the kinetic character of the group of hydrogens liberated upon oxidation of ferrocytochrome *varies with the progress of the exchange reaction.*

The converse experiment, reduction of ferricytochrome *c* during the course of its exchange, similarly indicates the kinetic heterogeneity of those peptide hydrogens which are affected by the redox state of the protein. Figure 4 shows that reduction of ferricytochrome shortly (1.5 min) after dissolving the protein in D_2O modifies the course of the kinetic curve from that of the control ferricytochrome, at 6 min, to an intermediate value, at 66 min, and finally to that characteristic of control ferrocytochrome, at 336 min. Later reduction, after 28 or 78 min, leads to similar, although less pronounced, effects, *i.e.*, the value for the control ferrocytochrome is approached but not completely attained within the period of observation. Thus, the effect of the reduced conformation in retarding exchange is evident until late in the time course when most of the available amide hydrogens have already exchanged.

These experiments demonstrate directly that oxidoreduction affects both rapidly and more slowly exchanging peptide hydrogens which are present in varying proportions as the exchange reaction proceeds. The nearly constant *net* difference of nine or ten hydrogens maintained between the oxidized and reduced cytochromes throughout the course of exchange would seem, therefore, to reflect a "steady state" sustained by a still larger and progressively changing population of different kinetic species. This is more readily visualized if the data from the curves for the two "modulated" redox states (Figures 3 and 4) are compared directly for the total number of hydrogens exchanging during a given time interval, *e.g.*, between 5 and 300 min (Table II). In the control ferri- and ferrocytochromes, a total of 15 and 16 exchanging hydrogens, respectively, can be identified during this interval, virtually the same for the two proteins. When, however, ferricytochrome is reduced during the first few minutes, only eight hydrogens exchange between 5 and 300 min; conversion into the more compact structure of the reduced protein retards exchange. In contrast, when ferrocytochrome is oxidized early in the experiment, 25 hydrogens ex-

TABLE II: Effect of Modulation of Redox Condition on Number of Peptide Hydrogens Exchanged.

Initial Redox Condn	Redox Modulation[a]	Hydrogens Exchanged/Mole (5–300 min)
Ferricytochrome	None	15
Ferrocytochrome	None	16
Ferricytochrome	Reduction	8
Ferrocytochrome	Oxidation	25

[a] Data calculated from Figures 3 and 4. Conditions as in Table I except that aliquots of the control ferricytochrome were reduced (with ascorbate) or the control ferrocytochrome were oxidized (with ferricyanide) *after* 1.5-min exposure to D_2O.

change in the subsequent comparable period; the more open structure of the oxidized protein exposes additional atoms. Hence, during the indicated interval, at least 17 hydrogens exchange differently in the two oxidation states of cytochrome (Table II). Structural modulation provides evidence that the difference in the two protein forms may be greater than apparent from the degree of displacement of the individual kinetic curves (Figure 2).

Discussion

Cytochromes, because of their ubiquity, their critical functional roles, and their distinctive spectra, have long been favored systems for investigation, and the properties of cytochrome *c*, the best characterized member of the terminal oxidation chain, have been intensively studied for more than 35 years (Keilin, 1930). Differences in the physical–chemical characteristics of the oxidized and reduced forms of this protein were recognized almost from the beginning of this period (Zeile and Ruter, 1933). Margoliash and Schejter (1966) have reviewed the large amount of experimental information on record which, although circumstantial, leaves little doubt that the conformation of the two oxidation states of cytochrome *c* differs.

The nature and magnitude of this difference is of special interest since it delineates *two* discrete thermodynamic and functional protein conformations arising from the same primary structure, a single peptide chain to which the heme is covalently bonded. Moreover, for cytochrome *c*, the circumstances which induce the altered conformation are nearly unique in their simplicity: the entire change in structure derives from a single, readily reversible chemical event, surrendering or absorbing one electron.

Determination of hydrogen exchange appears to be a particularly sensitive means for further exploration of this problem. It has been found that at least a portion of the hydrogens of most native globular proteins are restricted in their reactivity toward solvent water, *i.e.*, they undergo "slow" exchange in comparison with model oligopeptides, random coil polypeptides, and denatured proteins. Most such slowly exchanging members are thought to be peptide amide hydrogens and their relative abundance is related to the compactness of the molecule as defined by its secondary and tertiary structure (Hvidt and Nielsen, 1966). Changes in protein conformation which alter the degree of exposure of these hydrogens to solvent are reflected by changes in the kinetic exchange curves of hydrogen for deuterium.

During the past decade, a variety of techniques have been described for the measurements of the rates of hydrogen exchange (Lenormant and Blout, 1953; Linderstrøm-Lang, 1955; Hvidt and Linderstrøm-Lang, 1955; Blout *et al.*, 1961; Englander, 1963) and the relative advantages and results of different procedures have been reviewed (Schellman and Schellman, 1964; Harrington *et al.*, 1966; Hvidt and Nielsen, 1966; Englander, 1967). In the present investigation, we have employed the infrared spectrophotometric procedure (Blout *et al.*, 1961) which permits measurement of the rates of exchange of the peptide amide hydrogens only. This method is advantageous in terms of speed and simplicity and because the hydrogens determined are those likely to be of greatest interest for evaluating conformation. However, general application of this procedure may be limited by the requirement for high protein concentrations (demanding sufficiently high solubility), and the accuracy of the results is influenced by factors such as overlapping absorption of the carboxylate and amide II bands, temperature variations while the sample is in the infrared beam, definition of the correct absorption coefficients for the amide II band, and the appearance of artefacts during preparative lyophilization of the samples (Hvidt and Nielsen, 1966). Fortunately, many of the ambiguities encountered in the infrared method cancel out when, as in the present study, one compares the *apparent* exchange rates of two differing states of the same protein under identical conditions.

At pD 7.4, more than half the peptide amide hydrogens of either oxidized or reduced cytochrome *c* exchange very rapidly with deuterium (Figure 2 and Table I) at rates comparable with those observed for simple peptides and small model compounds (Nielsen, 1960; Bryan and Nielsen, 1960). These groups appear to be freely accessible to the solvent. Under the same conditions, about 20% of the amide hydrogens in both forms of the protein exchange slowly, over the ensuing 24 hr and, presumably, are occluded by conformational folding and/or intramolecular hydrogen bonding from effective contact with the solvent. At the end of 24 hr an unexchanged core of 10% remains in the oxidized protein while in the reduced form this value is 20%. These core hydrogens appear to indicate a relatively inert or shielded area in both oxidation states of the native protein since they exchange only after a much more prolonged exposure to solvent or after denaturation by heat or detergents.

The observed difference in H–D exchange between ferri- and ferrocytochrome *c* does not appear to arise from artefacts during preparation of the samples, a problem sometimes encountered in other systems (Hvidt and Nielsen, 1966). The possibility of partial denaturation of the oxidized protein during lyophilization has been eliminated, since reversal of the redox condition at the very initiation of exchange transforms the kinetic exchange curve to that characteristic of the opposite oxidation state (Table I).

The kinetic exchange curves of both redox states of the protein, while displaced from one another, follow an approximately parallel course throughout the exchange reaction. In accord with the early suggestions of Linderstrom-Lang (1955), each exchange curve can be considered to reflect the sum of the first-order decay functions of several classes of labile peptide hydrogens having differing rate constants of exchange and each class being of a discrete size. In these terms, the nearly parallel displacement of the exchange curves induced by oxidation–reduction of cytochrome *c* could result from: (1) transformation of a fixed number of peptide hydrogens from the most slowly to the most rapidly exchanging class (or *vice versa*), (2) a more or less uniform acceleration (or retardation) of the rates of exchange of all classes of peptide hydrogens, or (3) a combination of these effects. Only in the first case will the displacement between the two curves be an accurate gauge of the number of peptide hydrogens affected by the structural change; in the other instances, the displacement indicates but a steady-state difference resulting from a changing population of peptide hydrogens having variably altered rate constants.

Experimentally, precise resolution of the different kinetic classes of hydrogens is often difficult; thus, it may not be possible to distinguish between a change in number of groups involved as opposed to variations in rate constants of a fixed number of groups. For example, both of these alternatives can account for differences of the H–D exchange curves of yeast alcohol dehydrogenase and its complex with DPN^+ (Hvidt and Kägi, 1963; Hvidt and Nielsen, 1966). In cytochrome *c*, the oxidation state of the heme iron and, therefore, protein structure, can be shifted instantaneously and completely at any time during the course of exchange. This circumstance provides an unusual opportunity to probe at varying times the kinetic characteristics of the peptide hydrogens which are affected by the redox change and, thus, to differentiate between alternative interpretations. If oxidation of ferrocytochrome transforms a fixed number of hydrogens from the most slowly to the rapidly exchanging category, oxidation at any time during the course of exchange should free these nine or ten hydrogens virtually instantaneously; by the same token, reduction of ferricytochrome should not alter the progress of its kinetic exchange curve after the period of rapid early exchange is completed.

Figures 3 and 4 demonstrate that this is not the case. The number of hydrogens freed by oxidation of ferrocytochrome diminishes markedly as the experiment progresses, while reduction of ferricytochrome, even after the initial period, results in significant retardation of exchange of remaining amide hydrogens.

On this basis it is shown directly that the hydrogens which differ in oxidized and reduced cytochrome constitute several discrete kinetic classes manifested in varying proportions at different times of exchange: initially, a rapidly exchanging group is most prominent; as the exchange progresses, a difference in slowly exchanging species is also identified. At any point during the course of exchange an average or net difference of nine to ten hydrogens is observed between the two oxidation states, but the actual total is considerably larger (Table II). Indeed, studies of the effect of pH upon the exchange reaction (Kägi and Ulmer, 1968) indicate that as many as three completely separate kinetic classes of slowly exchanging hydrogens can be differentiated. Thus, the total number of peptide hydrogens affected by the structural alteration is likely several times the observed difference of nine or ten.

Current deuterium exchange procedures are capable of monitoring accurately but a fraction of all the potentially exchangeable hydrogens in a protein which might be affected by a conformational change (Hvidt, 1964). In the present investigation of cytochrome *c*, for example, only that portion of the peptide hydrogens exchanging relatively slowly can be evaluated by infrared spectroscopy. The redox-reversal experiments (Figures 3 and 4 and Table II) indicate that modulation of protein structure during the course of the exchange reaction may *enhance* discrimination of the number of hydrogens affected by a structural alteration, thus increasing the sensitivity of the technique. This observation would appear to have general significance for interpretation of hydrogen-exchange data in other proteins.

The present studies of cytochrome *c* disclose large differences in the hydrogen exchange of two conformational states of the same protein examined under identical experimental conditions. Hence, this general approach is analogous to that employed for investigation of the effect of cofactors or substrates on the structure of yeast alcohol dehydrogenase (Hvidt and Kägi, 1963), lactic dehydrogenase (DiSabato and Ottesen 1965), creatine phosphokinase (Lui and Cunningham, 1966), luciferase (DeLuca and Marsh, 1967), and aspartate aminotransferase (Torchinskii *et al.*, 1967). These studies indicate that hydrogen exchange can prove a most powerful tool for identification of small changes in conformation which are related to protein functional states, and, by the same token, should lead to a better understanding of the function of a protein in terms of its structural flexibility. On the same basis, hydrogen-exchange techniques will also likely prove useful in delineation of structural alterations during the activation of zymogens and consequent to the interactions of proteins with metal atoms, drugs, and other small molecules.

Acknowledgment

The authors are indebted to Dr. B. L. Vallee for many helpful discussions.

References

Benson, E. S., Hallaway, B. E., and Lumry, R. W. (1964), *J. Biol. Chem. 239*, 122.

Blout, E. R. (1964), *Biopolymers Symp. No. 1*, 397.

Blout, E. R., Delozé, C., and Asadourian, A. (1961), *J. Am. Chem. Soc. 83*, 1895.

Bryan, W. P., and Nielsen, S. O. (1960), *Biochim. Biophys. Acta 42*, 552.

Butt, W. D., and Keilin, D. (1962), *Proc. Roy. Soc. (London) B156*, 429.

DeLuca, M., and Marsh, M. (1967), *Arch. Biochem. Biophys. 121*, 233.

DiSabato, G., and Ottesen, M. (1965), *Biochemistry 4*, 422.

Ehrlich, G., and Sutherland, G. (1954), *J. Am. Chem. Soc. 76*, 5268.

Englander, S. W. (1963), *Biochemistry 2*, 798.

Englander, S. W. (1967), *in* Poly-α-Amino Acids, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, pp 339–367.

Fraser, R. D. B., and Price, W. (1952), *Nature 117*, 419.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Harrington, W. F., Josephs, R., and Segal, D. M. (1966), *Ann. Rev. Biochem. 35*, 633.

Horecker, B. L., and Kornberg, A. (1946), *J. Biol. Chem. 165*, 11.

Horecker, B. L., and Stannard, J. N. (1948), *J. Biol. Chem. 172*, 589.

Hvidt, A. (1963), *Compt. Rend. Trav. Lab. Carlsberg 33*, 475.

Hvidt, A. (1964), *Compt. Rend. Trav. Lab. Carlsberg 34*, 299.

Hvidt, A., Johansen, G., and Linderstrøm-Lang, K. (1960), *in* Analytical Methods of Protein Chemistry, Vol. II, Alexander, P., and Block, R. J., Ed., Oxford, Pergamon, pp 101–130.

Hvidt, A., and Kägi, J. H. R. (1963), *Compt. Rend. Trav. Lab. Carlsberg 33*, 497.

Hvidt, A., and Linderstrøm-Lang, K. (1955), *Biochim. Biophys. Acta 18*, 308.

Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 287.

Kägi, J. H. R., and Ulmer, D. D. (1968), *Biochemistry 7*, 2718 (this issue; following paper).

Keilin, D. (1930), *Proc. Roy. Soc. (London) B106*, 418.

Lenormant, H., and Blout, E. R. (1953), *Nature 172* 770.

Linderstrøm-Lang, K. (1955), Society of Biological Chemists, India, Souvenir, p 191. Reprinted in Linderstrøm-Lang, K. (1962), Selected Papers, Copenhagen, Danish Science Press, p 547.

Lui, N. S. T., and Cunningham, L. (1966), *Biochemistry 5*, 144.

Margoliash, E. and Frohwirt, N. (1959), *Biochem. J. 71*, 570.

Margoliash, E., Reichlin, M., and Nisonoff, A. (1967), *Science 158*, 531.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.

Matsubara, H., Ando, K., and Okunuki, K. (1965), *Proc. Japan. Acad. 41*, 408.

Miyazawa, T., Shimanouchi, T., and Mizushima, S. (1958), *J. Chem. Phys. 29*, 611.

Myer, Y. P., and Harbury, H. A. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1391.

Nielsen, S. O. (1960), *Biochim. Biophys. Acta 37*, 146.

Nozaki, M., Mizushima, H., Horio, T., and Okunuki, K. (1958), *J. Biochem. (Tokyo) 45*, 815.

Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K. (1957), *J. Biochem. (Tokyo) 44*, 453.

Schellman, J. A., and Schellman, C. (1964), *Proteins 2*, 1.

Theorell, H., and Åkesson, Å. (1941), *J. Am. Chem. Soc. 63*, 1804, 1812, 1818.

Torchinskii, Yu. M., Abaturov, L. V., Varshavskii, Ya. M., and Nezlin, R. S. (1967), *Mol. Biol. 1*, 603.

Ulmer, D. D. (1965), *Biochemistry 4*, 902.

Ulmer, D. D. (1966), *Biochemistry 5*, 1886.

Ulmer, D. D., and Kägi, J. H. R. (1967), *Federation Proc. 26*, 386.

Urry, D. W., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 2756.

Willumsen, L. (1966), *Biochim. Biophys. Acta 126*, 382.

Zeile, K., and Ruter, F. (1933), *Z. Physiol. Chem. 221*, 101.