Hydrogen–Deuterium Exchange of Cytochrome *c*. II. Effect of pH*

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ABSTRACT: The hydrogen–deuterium exchange of ferri- and ferrocytochrome *c* has been measured at intervals of pH from 1.5 to 12.0. A difference in exchange between the oxidized and reduced proteins (Ulmer, D. D., and Kägi, J. H. R. (1968), *Biochemistry 7*, 2710 (this issue; preceding paper) is evident even at high and low pH, indicating a broad range of pH stability for both redox conformations. Both oxidation states exhibit minimal exchange between pH 4 and 5. On the extreme acid side, pH 1.5, exchange is accelerated due to a conformational transition, confirmed by spectropolarimetry. On the alkaline side, no conformational changes are observed, and increasing exchange as pH rises appears to stem solely from hydroxyl ion catalysis of the rate-limiting step of the exchange reaction. Analysis of the kinetic exchange curves in terms of proposals advanced by Hvidt and Nielsen (Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 287) suggests that both ferri- and ferrocytochrome *c* exchange by an EX_2 mechanism; the minimum difference in conformational free energy between the two oxidation states is estimated at about 2 kcal/mole.

In model amide compounds, simple peptides, and polyamino acids, the exchange of hydrogen for deuterium is catalyzed by both hydroxyl and hydrogen ions (Nielsen, 1960; Klotz and Frank, 1962, 1965; Leichtling and Klotz, 1966). Similarly, the hydrogen-exchange rates of proteins are strongly pH dependent, as first noted with insulin (Hvidt and Linderstrøm-Lang, 1955) and since examined carefully in a number of other systems (Hvidt and Nielsen, 1966). The majority of proteins studied thus far appear to exchange peptide amide hydrogens most slowly at a pH of 5 or lower, while exchange increases markedly and progressively as the solvent becomes more basic. Because of this behavior, variations in pH permit modulation of the rates at which exchange reactions proceed and, as a consequence, may provide insight into exchange mechanisms in proteins (Hvidt and Nielsen, 1966).

A marked difference in hydrogen–deuterium exchange between ferri- and ferrocytochrome *c*, at neutrality, has been described (Ulmer and Kägi, 1968). In the present paper, we report the effect of varying pH upon exchange of the two redox forms.

The results indicate that the disparate exchange characteristics of the oxidized and reduced proteins are maintained over a broad range of hydrogen ion concentration. In terms of the model proposed by Linderstrøm-Lang (1955), cytochrome *c* is identified as a high-motility protein. In addition, analysis of the kinetic exchange curves of cytochrome *c* as a function of pH permits an estimate of a minimum difference in conformational free energy of the two oxidation states.

Methods and Materials

Preparation of samples and infrared spectrophotometric measurements of hydrogen–deuterium exchange were carried out as described previously (Ulmer and Kägi, 1968).

DCl (38% in D_2O) and NaOD (40% in D_2O) were purchased from Merck Sharp and Dohme of Canada Ltd., Montreal. Protein solutions of alkaline or acid pH were obtained by adding, to protein samples lyophilized at neutral pH, measured volumes of NaOD, in D_2O, or DCl, in D_2O, to D_2O buffered with neutral sodium phosphate. The absorption of D_2O solutions in the infrared regions employed is increased at both extremes of pH. Therefore, suitable blank corrections to the amide bands of the protein solution at each pH were obtained by constructing curves of absorbancy *vs.* pH. The appropriate correction value was selected after direct measurement of the pH of the protein solution upon completion of the experiment.

Potentiometric measurements in either H_2O or D_2O solutions were made with a Radiometer pH meter equipped with a general purpose glass electrode or with a Beckman microglass electrode and standardized for pH measurements in H_2O solutions. pH values were converted into pD as recommended by Glasoe and Long (1960) and Mikkelsen and Nielsen (1960), pD = pH (meter reading) + 0.40, and the validity of the conversion is assumed to be applicable throughout the range of pH employed. Values measured in strongly alkaline solutions were corrected for the presence of sodium ions.

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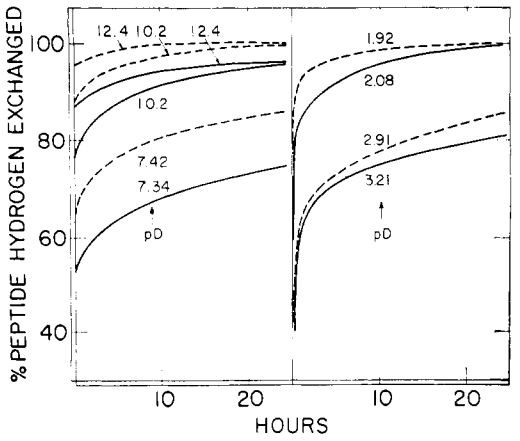

FIGURE 1: Effect of pD (pH + 0.40) on the time course of exchange of hydrogen for deuterium in oxidized and reduced cytochrome *c*. The per cent peptide hydrogens exchanged, measured by infrared spectrophotometry, is plotted against time at alkaline (on the left) and acid (on the right) pD. Both the reduced (———) and oxidized (- - - -) proteins exchange more rapidly at extremes of pD. Conditions: cytochrome *c*, 4.9 × 10^{-3} M, in 0.11 M sodium phosphate at the indicated pD (see Methods).

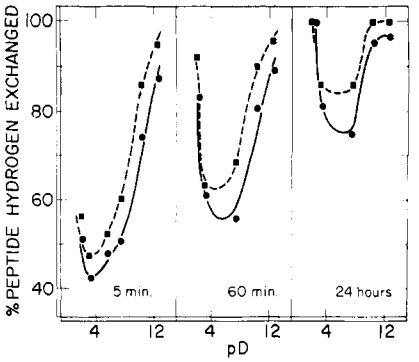

FIGURE 2: Effect of pD (pH + 0.40) on the extent of exchange of hydrogen for deuterium in oxidized and reduced cytochrome *c*, at different time intervals. The per cent peptide hydrogens exchanged in 5 min, 60 min, and 24 hr is plotted against pD for both oxidized (- - - -) and reduced (———) cytochrome. For both oxidation states, minimum exchange is observed between pD 4 and 5. Exchange is accelerated by either higher or lower pD, but the effect of acid is less pronounced early in the time course. Conditions: as in Figure 1, except exchange at pD 5 measured in 0.1 M sodium cacodylate.

Optical rotatory dispersion was measured in a Cary Model 60 recording spectropolarimeter as described previously (Ulmer, 1965, 1966). Cells with fused-quartz end plates and 0.1–0.2-mm path lengths were employed using protein concentrations of 0.2–0.25 mg/ml. Aliquots from a single stock solution of ferricytochrome *c*, in 0.1 M sodium phosphate buffer, were titrated to the required pH and then diluted to identical final protein concentrations, as determined spectrophotometrically on the sample at pH 7.0 after reduction with ascorbate (Margoliash and Frohwirt, 1959).

Results

The kinetic exchange curves of ferri- and ferrocytochrome *c* were examined over a range in pH from approximately 1.5 to 12 (pD 1.9–12.4) (Figure 1). To facilitate visualization of the data, the number of peptide hydrogens exchanged at each pH studied, after three representative intervals, are plotted in Figure 2. Notably, the difference between oxidized and reduced proteins in number of exchangeable amide hydrogens (Ulmer and Kägi, 1968) is maintained until late in the time course, except at the very extremes of pH. Thus, a broad range of pH stability exists for the structural parameters which distinguish the two conformational states. However, the magnitude of the difference between the two is smaller in acid media than in alkali, perhaps due to more rapid autoxidation of the ferroprotein at low pH.

For both oxidation states of the protein, minimum exchange is observed between pH 4 and 5. Either higher or lower pH increases the degree of exchange for both redox states at all time intervals shown. However, with both oxidized and reduced cytochrome, the form of the kinetic exchange curve at extreme acid pH differs markedly from that at the alkaline extreme. This is best seen by comparing the *early* time course of exchange of samples measured near neutrality (pD 7.4) with those determined at pD 1.9 and 12.4 (Figure 3). At alkaline pD the number of *rapidly* exchanging amide hydrogens (those exchanging within 5 min) is sharply increased in comparison with the values at pD 7.4; thus, at pD 12.4, exchange is nearly complete within the time required for the first measurement. In contrast, at acid pD (1.9), the number of hydrogens exchanging within the first 5 min is considerably smaller than is observed at pD 7.4 (Figure 3). Nevertheless, within 15 min, the total exchange at acid pD reaches and then surpasses that at neutrality; within 2 hr, at pD 1.9, exchange is virtually complete. Thus, at low pD, there are fewer hydrogens which exchange with the *most* rapid rates; however, most of those groups remaining after the first few min-

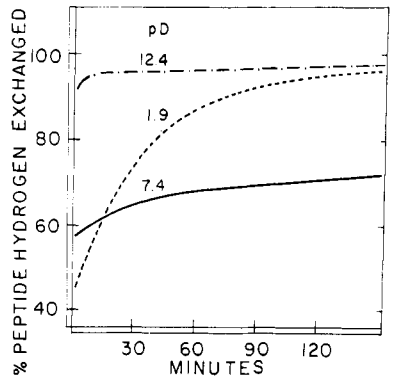

FIGURE 3: Effect of pD (pH + 0.40) on the early time course of exchange of hydrogen for deuterium in ferrocytochrome *c*. The per cent peptide hydrogens exchanged is plotted against time (redrawn from Figure 1). At pD 12.4, the per cent of hydrogens exchanging in the first few minutes is markedly increased compared with pD 7.4. At pD 1.9, however, fewer hydrogens exchange in the first few minutes than at pD 7.4 but, within 15 min, exchange accelerates to reach and then surpass that at neutrality. Conditions are as in Figure 1.

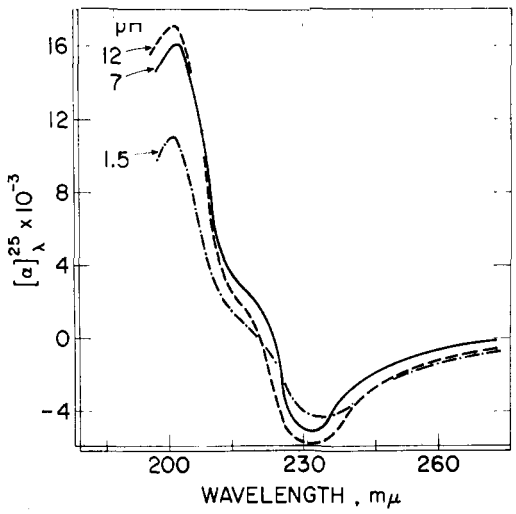

FIGURE 4: Intrinsic Cotton effect of ferricytochrome *c* at pH 12, 7, and 1.5. Specific rotation, $[\alpha]^{25}\lambda$, is plotted against wavelength. The magnitude of the Cotton effect is similar at pH 12 and 7, but significantly decreased at pH 1.5. Conditions: 0.2 mg/ml of protein in 0.1 M sodium phosphate at the indicated pH. Substitution of D_2O for H_2O as solvent did not significantly alter these results.

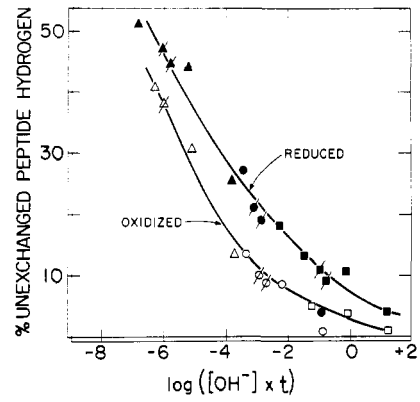

FIGURE 5: Effect of pH on the hydrogen–deuterium exchange of oxidized and reduced cytochrome *c*. The per cent unexchanged peptide hydrogens is plotted against the logarithm of the product of the hydroxyl ion concentration and the duration of the exchange reaction (Willumsen, 1966). At pH values greater than 5, the data for both oxidation states of cytochrome describe a monotonically decreasing function and the curves are separated by about 1.5 log units. The data are taken from those shown in Figure 1. Each set of symbols represents measurements at a single pH.

utes exchange faster than their presumable counterparts at pD 7.4.

Variation in pH may influence hydrogen exchange of proteins solely by catalysis of the rate-limiting step in the exchange reaction; however, in addition to affecting rate constants, changes in pH may also induce alterations in protein conformation (see Discussion). Therefore, evaluation of protein structure by an independent method is helpful for interpretation of the effects of pH upon exchange. For this reason, the influence of acid and alkaline pH on the structure of cytochrome *c* was examined by means of spectropolarimetry.

Figure 4 indicates the short-wavelength optical rotatory dispersion of ferricytochrome *c* at the extremes of pH employed for the exchange experiments. As evidenced by the magnitude of the intrinsic Cotton effect,[1] the conformation of the protein is well maintained at pH 12 as compared with pH 7, consistent with previous observations (Ulmer, 1966). At pH 1.5, however, there is a significant *decrease* in the magnitude of the intrinsic Cotton effect, indicative of altered protein structure. Thus, it seems likely that at extreme acid pH a conformational transition in the protein could play a significant role in generating the highly distinctive exchange curve (Figure 3). Conversely, the absence of a conformational transition on the alkaline side suggests that structural variations are not involved in the acceleration of exchange above pH 4–5. Presumably, variations in exchange rates of cytochrome *c* with increasing pH should be attributable primarily to alterations of the rate-limiting constants.

Hvidt (1964; Hvidt and Nielsen, 1966) has elaborated a mechanism for the pH dependence of the hydrogen-exchange reaction of proteins (see Discussion) in which the acid- and base-catalyzed exchange of exposed peptide groups, as in model compounds, functions as the rate-limiting step (EX_2 mechanism). A characteristic feature of this mechanism is that, in the absence of a pH-induced conformational transition, the progress of the exchange reaction (above pH 4) should describe a composite first-order decay function of the product of time and hydroxyl ion concentration (*vide infra*). This proposition can be tested conveniently by plotting the proportion of unexchanged peptide hydrogens of the protein against the logarithm of the product of hydroxyl ion concentration and the duration of the exchange reaction (Willumsen, 1966). Pertinent portions of the data from Figures 1 and 2 are replotted in this manner in Figure 5. Notably, the data obtained at pH values greater than 5 describe a monotonically decreasing function precisely as would be anticipated if, as pH is raised, exchange accelerates solely due to hydroxyl ion catalysis. This is evident for both oxidation states of the protein.

This representation of the data reveals several additional features pertinent to the present system. The curves for both oxidized and reduced cytochrome span a broad range with reference to the abscissa (log ($[OH^-]t$)), in accord with the wide variations known for the exchange rates of peptide hydrogens in proteins (Hvidt and Nielsen, 1966). First-order decay functions theoretically decrease from 95 to 5% of the initial value over less than two log units; therefore, the spread of the experimental curves over 6 log units indicates the participation of at least three separate classes of slowly exchanging peptide hydrogens in cytochrome *c*, and these must differ as much as 10,000-fold in bimolecular rate constants.

Moreover, the curves for the oxidized and reduced proteins follow a nearly parallel course, separated by about 1.5 log units. This displacement is equivalent to an average 30-fold difference in the bimolecular

[1] As defined in footnote 1 of Ulmer and Kägi (1968).

exchange rate constants between the two oxidation states and provides the basis for a quantitative estimate of the difference in compactness of the two molecules.

Discussion

Measurements at pH 7.0 have revealed a marked difference in the rates of hydrogen–deuterium exchange of ferri- and ferrocytochrome *c* indicating an oxidoreduction-linked change in protein conformation (Ulmer and Kägi, 1968). Evaluation of exchange characteristics over a range of hydrogen ion concentration provides an estimate of the pH stability of the features which maintain this conformational difference. In addition, studies of exchange as a function of pH serve to elucidate possible mechanisms by which hydrogens in proteins are exchanged and, thereby, may provide insight into the dynamic features of macromolecular structure in the two redox states.

The present data may be analyzed in terms of the motility model of Linderstrøm-Lang (Linderstrøm-Lang, 1955; Berger and Linderstrøm-Lang, 1957; Hvidt, 1964; Hvidt and Nielsen, 1966) in which proteins in aqueous solution are considered to undergo continuous fluctuations of the secondary and tertiary structure about an "average" native conformation. If it is assumed that, at chemical equilibrium, each peptide hydrogen, m, alternates between either a totally shielded conformation, N_m, where no exchange with the solvent takes place, and an open conformation, I_m, where the peptide group is fully exposed to solvent water and exchanges its hydrogens with a first-order rate constant, k_3, similar to that found in oligopeptides, the exchange mechanism can be written as eq 1, where

$$N_m \underset{k_{2,m}}{\overset{k_{1,m}}{\rightleftharpoons}} I_m \xrightarrow{k_3} \text{exchange} \qquad (1)$$

$k_{1,m}$ and $k_{2,m}$ are the rate constants governing the transconformational reaction. Depending upon the relative magnitude of the rate constants, two limiting first-order rate expressions, referred to as EX_1 and EX_2 mechanisms, can be derived from this scheme for each peptide hydrogen in the protein (Hvidt and Nielsen, 1966). The first is thought to predominate in proteins with low motility ($k_3 \gg k_{2,m} \gg k_{1,m}$) and has an exchange rate constant, K_m, equal to that of the transconformational reaction $N_m \rightarrow I_m$, *i.e.*, $K_m = k_{1,m}$. In the case of the EX_2 mechanism, found in proteins of high motility ($k_{2,m} \gg k_{1,m} \gg k_3$), the over-all rate depends both upon k_3 and on the relative proportion of the open and shielded conformations present, *i.e.*, $K_m = (k_{1,m}/k_{2,m})k_3$. Thus, depending upon which step is rate limiting, entirely different information will be obtained from hydrogen–deuterium-exchange measurements.

The proposed mechanisms may be differentiated experimentally on the basis of the effect upon exchange of variations in pH or temperature (Hvidt and Nielsen, 1966). Studies on model compounds show that k_3 increases exponentially on going toward the extremes of pH, indicating acid and base catalysis (Bryan and Nielsen, 1960; Klotz and Frank, 1965). Exchange rates of a protein obeying the EX_2 mechanism should vary with pH in an analogous fashion, while no such systematic pH dependence would be anticipated for cases where the EX_1 mechanism is operative. However, in the EX_2 mode, the exchange rate also depends upon the transconformational equilibrium constant, $k_{1,m}/k_{2,m}$; hence, a pH-linked conformational alteration may *also* register as a systematic variation in exchange with pH. For this reason the absence of a pH-dependent conformational change must be ascertained before the two different kinetic modes can be differentiated unambiguously. The effects of pH upon the exchange properties of cytochrome *c* can be viewed in the light of these considerations.

The rates of H–D exchange in both oxidized and reduced cytochrome *c* are markedly increased by either raising or lowering pH (Figures 1–3). On the alkaline side, the progressive acceleration of exchange resembles that found previously for insulin (Hvidt and Linderstrøm-Lang, 1955), ribonuclease (Hvidt, 1955; Stracher, 1960; Schildkraut and Scheraga, 1960; Blout *et al.*, 1961; Englander, 1963), and lysozyme (Hvidt, 1963; Hvidt and Kanarek, 1963), suggesting that the EX_2 mode may be operative, as is presumed for these systems (Hvidt and Nielsen, 1966). On the other hand, a minimum between pH 4 and 5 and acceleration of exchange on the acid side were not seen with these other proteins. This suggests that such changes may represent a more specific feature of the cytochrome *c* system and indicates that the factors determining the exchange reaction in different pH regions may vary. This is confirmed both by spectropolarimetry and by a further analysis of the exchange curves.

Previous spectropolarimetric studies have shown that the *intrinsic* Cotton effects[1] of both redox states of the protein vary little over a range of pH from 3 to 12 (Myer and Harbury, 1965; Urry, 1965; Ulmer, 1966). The conformational stability over this range of values was verified in the present study; however, under still more acid conditions, significant denaturation of the protein does occur as evidenced by a decrease in magnitude of the intrinsic Cotton effect, at pH 1.5, to only two-thirds the value found at neutral pH (Figure 4). A conformational change in cytochrome *c* at acid pH has also been reported recently based upon viscosimetric studies (Bull and Breese, 1966) and upon investigations of circular dichroism (Myer, 1968).

The altered structural form of the protein in acid appears to generate a highly distinctive kinetic exchange curve (Figure 3). At pD 1.9 the extent of exchange at the time of the initial observations is decreased compared with that observed at neutrality; after the first few minutes, however, despite the lower early values, the extent of exchange has increased markedly and, within an hour, equals and then exceeds that at pD 7.4. In this instance one cannot distinguish the relative contributions to the exchange curve of hydrogen ion catalysis as opposed to conformational alteration. Benson *et al.* (1964) have reported a similarly distinctive kinetic curve at acid pH for bovine serum albumin; below pH 4, the initial degree of exchange of albumin is low compared with that at neutrality but, later in the reaction,

increases so as to surpass that at pH 7. Albumin is known to undergo a conformation transition below pH 4 (Foster, 1960) and it is possible that "anomalous" kinetic curves, similar to those engendered by cytochrome *c* and albumin at acid pH, may serve to identify conformational transitions in other proteins.[2]

Over the range of alkaline pH employed, no alteration in protein conformation is identified by means of spectropolarimetry. This suggests that increased exchange as pH rises is likely due primarily to hydroxyl ion catalysis of the rate-limiting step. Hence, quantitative evaluation of the pH dependency over this region should permit identification of the presumable kinetic mechanism of exchange.

As suggested by Willumsen (1966), this may be accomplished by graphical analysis of the exchange data in terms of Hvidt's proposals (Hvidt, 1964).[3] When the experimental values of p, at varying pH and times of incubation with D_2O, are plotted *vs.* log ($[OH^-] \times t$) according to Willumsen's formulation, the data for both oxidized and reduced cytochromes describe a monotonically decreasing function (Figure 5). This graphical result confirms the absence of any conformational change with pH throughout the neutral and alkaline range examined and reinforces the interpretation that pH-dependent acceleration of exchange can be accounted for quantitatively by OH^- catalysis. Thus, the exchange of both redox states appears to proceed by the EX_2 mechanism. Comparable graphical analysis indicating that ovalbumin exchanges by the same mechanism has been reported recently (Willumsen, 1967), while in the case of heavy meromyosin, the only other protein analyzed in this fashion, the effects of pH on exchange are more complex (Willumsen, 1966).

An additional feature of special pertinance to the cytochrome system becomes apparent when the data are plotted as a function of ($t \times [OH^-]$). From Willumsen's derivation[3] it is evident that differences in the bimolecular exchange function between two proteins can arise only from a change in the effective rate constants, $(k_{1,m}/k_{2,m}) \times C \times 10^{T/20}$. Since the constant C is presumed to be identical for all proteins the differences in exchange between oxidized and reduced cytochromes must reflect a change in the N–I equilibria (where N represents the nonexchanging, shielded, and I, the exchanging or open conformations of peptide hydrogens). Hence, the displacement of the exchange curves of the two forms (Figure 5) provides thermodynamic corroboration of their differences in conformation in terms of the assumed model. The curve for the oxidized cytochrome is positioned to the left of that for the reduced; this indicates that oxidation shifts the N–I equilibria of the affected peptide groups from a closed to a more open structure, characterized by a higher value for $k_{1,m}/k_{2,m}$.[3]

The *extent* to which the curves of the two proteins are displaced from one another (Figure 5) provides a measure of the difference in conformational free energy between the N–I equilibra of the peptide hydrogens exchanging at a given value of p; the nearly parallel course of the two curves suggests a more or less uniform energetic difference between equally ranking though not necessarily identical peptide groups of the reduced and oxidized proteins. From the average displacement of the curves of about 1.5 log units it is possible, thereby, to estimate an average difference in energy for all the N–I equilibria of the two redox forms of approximately 2 kcal/mole. This figure is a minimum value and applies to the hypothetical case in which the redox change affects uniformly all peptide hydrogens of the protein. To the extent that this condition is not met in the present instance, the difference in energy would be correspondingly larger.

Estimates of the free-energy difference between the two conformational forms of cytochrome *c* have been made previously on the basis of quite different considerations. Early spectroscopic investigations of this protein led to the suggestion that the heme was buried in a crevice (for review, see Margoliash and Schejter, 1966). To account for the reactivity of the heme iron with external ligands the so-called crevice equations were introduced describing equilibria between a predominant, nonreactive "closed" crevice and a reactive, "open" crevice conformation in *each* oxidation state (George and Lyster, 1958a,b; George *et al.*, 1963). Operationally, the proposed crevice equilibria play a role similar to the N–I equilibria discussed in the present work. Both formulations postulate fluctuations between reactive and nonreactive states of protein structure, *i.e.*, conformational motility (Linderstrøm-Lang and Schellman, 1959). Moreover, both are similarly affected by oxidoreduction, *i.e.*, reduction shifts both types of equilibria toward the less reactive, more

[2] We have recently observed a similar "anomalous" kinetic exchange curve at acid pH for egg conalbumin.

[3] The complete exchange equation for a protein is given by the sum of the first-order expressions for each peptide hydrogen in the molecule. In the most likely case, where all groups exchange by the EX_2 mode, the proportion, p, of unexchanged peptide hydrogens at a given time, t, is

$$p = \frac{1}{h}\sum_{m}^{h} e^{-\frac{k_{1,m}}{k_{2,m}} k_3 t}$$

where m represents any peptide hydrogen, h their total number, $k_{1,m}/k_{2,m}$, the transconformational equilibrium constant, and k_3, the first-order rate constant of the acid- and base-catalyzed exchange with H_2O in fully exposed peptide groups. (This expression is valid, of course, only as long as k_3 remains rate limiting (see Discussion). At extremes of pH where k_3 is large this condition may no longer be fulfilled and the exchange more likely proceeds by the EX_1 mode.) Assuming k_3 to vary with hydroxyl ion concentration and temperature in the same fashion as in model compounds, *i.e.*, $k_3 = C \times 10^{T/20}[OH^-]$ (above pH 4) (Bryan and Nielsen, 1960), substitution yields the bimolecular exchange equation

$$p = \frac{1}{h}\sum_{m}^{h} e^{-\frac{k_{1,m}}{k_{2,m}} \times C \times 10^{T/20} \times [OH^-] \times t}$$

where C is a pH-independent constant, T the temperature in degrees centrigrade, and where the product, $(k_{1,m}/k_{2,m}) \times C \times 10^{T/20}$, represents the bimolecular rate constant for the base-catalyzed exchange of the mth peptide hydrogen. Presuming that, in the case of cytochrome *c*, the transconformational equilibria are independent of pH, as suggested by spectropolarimetry, it is apparent that p should vary as a continuous monotonic function of the product ($[OH^-] \times t$) (Willumsen, 1967).

stable conformation. Although the present data cannot establish any physical correspondence between the crevice equilibria and the various N–I equilibria, it is possible that both reflect, in a more or less parallel and equivalent fashion, the same dynamic alterations in the structure of cytochrome *c*. If, indeed, such a relationship between the equilibria should exist, it might permit a comparison of certain energy and redox parameters.

For example, investigations of heme–ligand reactions have shown that the free energy of the crevice-closing reaction in ferricytochrome *c*, ΔF_{cr}, has a value of between −3 and −4 kcal/mole (Margoliash and Schejter, 1966), while equivalent *direct* measurements of the strength of the ferrocytochrome *c* crevice have not been possible because of experimental limitations. Addition of the average value of the free energy difference of the N–I equilibria, $\Delta F_{N-I,red} - \Delta F_{N-I,ox}$, to ΔF_{cr} of ferricytochrome *c* would indicate that ΔF_{cr} of the reduced protein is at least −5 to −6 kcal/mole.

On the same basis, the minimum free-energy change derived from the hydrogen-exchange measurements may be employed to obtain an independent estimate of the maximum value of the redox potential of the open-crevice conformation of cytochrome *c*. The value calculated in this manner is approximately +0.170 V.[4] This is reasonably close to that for myoglobin, + 0.120 V, a realistic open-crevice model used in the estimation of other crevice parameters (Margoliash and Schejter, 1966). The coincidence of the numerical values seems relatively good in view of the many assumptions involved in the diverse approaches and lends some credence to the hypothesis that shifts in the crevice equilibria and the N–I equilibria are parallel manifestations of the redox-linked conformational transition of cytochrome *c*. As a corollary, these observations may indicate that the peptide hydrogens which are altered in their exchange characteristics by oxidoreduction are, in fact, those which are localized in or about the cytochrome *c* crevice. X-Ray crystallographic studies in progress (Dickerson *et al.*, 1967) will doubtless be helpful in indicating the number of groups potentially affected by the redox change and the plausability of these speculations. In even more immediate terms, it will be of great interest to determine in other systems to what degree thermodynamic information gained from hydrogen-exchange studies bears a relationship to results obtained by more conventional analysis.

Acknowledgment

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[4] At 25° the redox potentials, E_0', of the open and closed crevice forms to cytochrome *c* are related by the equation $E_0'_{,open} = E_0'_{,closed} + (1/23.1)(\Delta F_{cr,red} - \Delta F_{cr,ox})$, where $\Delta F_{cr,red} - \Delta F_{cr,ox}$ is the free-energy difference of the crevice equilibria in the two oxidation states expressed in kilocalories per mole. $E_0'_{,closed}$ can be assumed to coincide with the experimental value of +0.260 V (Margoliash and Schejter, 1966).

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Kinetic Studies of the Enzymatic Dopamine β-Hydroxylation Reaction*

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ABSTRACT: Acetate can replace fumarate in the stimulation of dopamine β-hydroxylase activity. Increased oxygen concentration enhances the enzymatic activity and can replace fumarate in the stimulation of the enzymatic β hydroxylation. Fumarate and oxygen stimulate the enzymatic activity at low substrate (RH) concentrations and both change the K_M of the substrate but not the V_{max}. Fumarate facilitates the interaction of the reduced enzyme intermediate with oxygen and most likely induces a conformational change of the enzyme. The initial velocity patterns are consistent with a mechanism in which the binding of the first substrate (ascorbate) to the enzyme is followed by the release of the product (dehydroascorbate) before a second substrate can react ("Ping-Pong" mechanism). The subsequent substrates (RH and O_2) add to the enzyme before either product is released. The data also suggest that regardless of which of these two substrates (RH or O_2) adds first, both steps are in a rapid equilibrium and the interconversion of the central ternary complexes most likely represents the rate-limiting step in the over-all β-hydroxylation reaction.

Dopamine β-hydroxylase is a mixed-function oxidase that catalyzes the conversion of 3,4-dihydroxyphenylethylamine into norepinephrine according to reaction I (Levin *et al.*, 1960).

$$\text{dopamine} + \text{ascorbate} + O_2 \xrightarrow{\text{fumarate}} \text{norepinephrine} + H_2O + \text{dehydroascorbate} \quad \text{(I)}$$

The enzyme is not specific for dopamine and catalyzes the β hydroxylation of many analogs of phenylethylamine (*e.g.*, tyramine) (Goldstein and Contrera, 1962; Levin and Kaufman, 1961; Creveling *et al.*, 1962). Fumarate and certain other dicarboxylic acids stimulate the hydroxylation reaction (Levin *et al.*, 1960) by a mechanism which is still obscure. The hydroxylating enzyme is a copper protein, and it was shown that part of the protein-bound copper undergoes cyclic reduction and oxidation during the over-all hydroxylation reaction (Goldstein *et al.*, 1965; Blumberg *et al.*, 1965; Friedman and Kaufman, 1965). The cupric copper of the enzyme is reduced by ascorbate and partially reoxidized when the reduced enzyme reacts with dopamine and oxygen. It was shown that the cupric copper of the enzyme can also be reduced by other reducing agents such as cysteine or reduced pteridine (Goldstein, 1966; Goldstein and Joh, 1967a). The reduced enzyme intermediate reacts with the substrate to give the hydroxylated product (Friedman and Kaufman, 1965). In the present study we have shown that the enzymatic activity is not only stimulated by fumarate but acetate also stimulates the β-hydroxylation reaction. We have also shown that increased oxygen concentration enhances the enzymatic activity and can replace fumarate in the stimulation of the enzymatic β hydroxylation. The results obtained with kinetic studies provide further information on the mechanism of the enzymatic β hydroxylation. Some of these results have been presented in preliminary reports (Goldstein and Joh, 1967b; Goldstein *et al.*, 1967).

Methods

Dopamine β-hydroxylase was prepared as previously described (Levin *et al.*, 1960). The enzyme was purified to the second DEAE column eluate stage as previously described in procedure A (Goldstein *et al.*, 1965). In some experiments an essentially homogeneous enzyme preparation was used. This enzyme preparation was prepared by the procedure of Friedman and Kaufman (1965). When dopamine was used as a substrate the enzyme activity was assayed fluorimetrically (Von

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