Unfolding of swine pepsinogen to a cross-linked random coil, as measured by spectrophotometric titration of tyrosine residues after pH jumps to above pH 11, is shown to be followed by slow reversible reactions. Absorbance studies show further small changes in the ionization of tyrosine residues. Double pH jump experiments indicate that the unfolded protein changes from an initial form that can refold rapidly to a mixture of forms which refold slowly. Both of these reactions in the unfolded protein are distinct from irreversible denaturation and show thermodynamic features which resemble cis-trans isomerization of proline residues. The inactive folded form of the zymogen, which occurs above pH 8 and was previously identified as an intermediate in unfolding by urea (McPhie, P. (1980) J. Biol. Chem. 255, 4048-4052) unfolds much more rapidly above pH 11 than molecules originating at neutral pH. The initial product of this rapid unfolding is found to refold slowly.

These slowly refolding forms show complex behavior on returning to neutral pH. Absorbance changes disclose the rapid formation of partially folded forms of the protein, which must undergo further slow reactions to reach the native state. These results are interpreted to show that native pepsinogen is in rapid equilibrium with the initial unfolded form. Slowly folding forms are produced by proline isomerization after unfolding. The partially folded forms detected during refolding do not lie on the most direct route of folding, but are trapped by proline isomers, which differ from those found in the native protein.

Above pH 8, swine pepsinogen is changed to a form which cannot activate itself to pepsin in acid solution and is precipitated at high concentrations of salt (1). However, the molecule is not denatured since the large changes in optical rotation or viscosity characteristic of protein unfolding reactions are only seen above pH 9 (2, 3). Thus, the two overlapping structural transitions in the molecule at alkaline pH involving three forms of the protein are inactivation, with minor alterations in physical properties which changes the native zymogen (N) to a folded inactive form (I), and unfolding to a cross-linked random coil form (U). Kinetic studies showed that the inactivation reaction is slow and that its product unfolds much more rapidly above pH 10 or in high concentrations of urea than molecules originating at neutrality. Furthermore, the rate of the slowest step in the refolding of pepsinogen from the random coil form was shown to be equal to the rate of reversal of the alkaline inactivation (4, 5). This suggested that the alkali-inactivated form is a stable intermediate in the unfolding and refolding of pepsinogen between the native and the unfolded random coil forms of the protein.

\[ N \xrightarrow{\text{slow}} \text{fast} \xrightarrow{U} \]

Determination of the enthalpies of activation of the rates of unfolding and refolding, in the presence of urea, indicated that the slow step involves the isomerization of proline residues in the protein (5). This was a puzzling conclusion, since this reaction is postulated to occur after unfolding, rather than as the initial step in the reaction (6, 7). Consequently further studies were undertaken on the nature and origin of this inactive form of pepsinogen (I) in the hope of producing a more detailed model of the folding mechanism. Its behavior was compared with that of the native protein (N) after pH jumps to unfolding and refolding conditions, using two probes, absorbance and potential pepsin activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Swine pepsinogen (Lot PG 303 608) and bovine hemoglobin (Lot HB 36H 756) were from Worthington. All other reagents were analytical grade or equivalent. Stock solutions of pepsinogen were made up in distilled water at concentrations of 2 to 4 mg/ml and pH values between 6 and 6.5. To study the properties of the inactive form of pepsinogen (I), solutions were adjusted to pH 10 by the addition of small volumes of 0.1 N sodium hydroxide with stirring and equilibrated for 15 min (5). Previous results on the pH dependence of the alkaline transitions indicated that 90% of the protein would be present as the inactive form (I) under these conditions of temperature and ionic strength (4, 5).

**Methods**

**Kinetics of Unfolding**—Initial experiments on unfolding in the pH range of 10 to 12 were made in an Amino-Morrow stopped flow apparatus, as described elsewhere (4). Equal volumes of pepsinogen solutions and 0.1 M sodium bicarbonate buffers were mixed, and unfolding was followed by the large transmission change at 295 nm caused by the ionization of tyrosine residues in the protein. Later experiments were performed in a Cary 15 spectrophotometer using the 0.0-0.1 absorbance scale. Equal volumes of pepsinogen solutions and bicarbonate buffer were mixed in the cuvette (mixing time of 10 s), or small volumes of 1 N sodium hydroxide solution were added to pepsinogen solutions using an Add-A-Mixer from Precision Cells Inc. (mixing time of 3 s). Absorbance changes were followed at 295 nm.

**Kinetics of refolding**—Aliquots of 1 N sodium hydroxide (5 μl) were added to 500 μl of pepsinogen solution in a cuvette and bleded on a Vortex mixer. After a carefully measured interval, 500 μl of 0.1 M sodium phosphate buffer, pH 7, were added to the cuvette, and the time course of the resulting absorbance increase at 287 nm was measured. The mixing time of these experiments was 10 s. The procedure was repeated, varying the delay time between the additions of sodium hydroxide and of phosphate buffer.
Reappearance of Potential Pepsin Activity during Refolding—The experimental procedure was identical with that described above. At intervals after the addition of phosphate buffer, 50-μl samples were removed from the cuvette and assayed for potential proteolytic activity in a 25-mg/ml solution of hemoglobin, pH 1.8, as described previously (8). All other techniques were described before (4, 5). Unless stated otherwise, all experiments were performed at 25 °C.

RESULTS

Absorbance Changes on Unfolding—The results obtained using the stopped flow apparatus were in good agreement with those published previously (4). Mixing a neutral solution of pepsinogen with sodium bicarbonate buffers was found to produce large increases in absorbance at 295 nm, resulting from ionization of tyrosine residues in the protein. The total change in absorbance increased sigmoidally with the final pH in the range of 10 to 12. At all pH values, half of the change occurred slowly. The characteristics of this change were identical with those determined in pH jumps starting from the native protein (N).

Variation of Potential Pepsin Activity—Other investigators have often seen slow changes in the absorbance spectra of proteins at high pH and have ascribed them to irreversible denaturation (e.g., Ref. 8). Consequently, it seemed important to determine the effect of extended exposure to unfolding conditions on the potential proteolytic activity of the protein. Samples of a neutral solution of pepsinogen (500 μl) were jumped to pH 11.5 by the addition of 5 μl of 1 M sodium hydroxide. After a carefully timed interval, an equal volume of 0.1 M sodium phosphate buffer, pH 7, was added to give a final pH of 7.3. The intermediate exposure to neutral pH allowed reversibly unfolded protein to regain the native conformation. Aliquots of the neutralized solution were assayed at various times after the pH drop for their potential pepsin activity as a measure of the recovery of native protein (N). Irreversible denaturation could not be detected in this way after 5 min at pH 11.5 although slight losses of potential activity occurred after longer periods. However, incubation at pH 11.5 for 5 min had marked effects on the kinetics of the reappearance of potential pepsin activity (Fig. 2). Thus, pepsinogen exposed for 10 s at pH 11.5 was found to be 83% in the native form (N) only 10 s after returning to neutrality. Exposure for 5 min at pH 11.5 produced protein which showed only 14% of its potential activity 10 s after the pH drop. In all cases the remainder of the activity was regained slowly.

To study the behavior of the inactive form of pepsinogen (I), solutions of the protein were equilibrated at pH 10 for 15 min and then jumped to pH 11.5 by the addition of 4 μl of 1 M sodium hydroxide to 500 μl of solution. In this case, extended exposure to pH 11.5 had a less marked effect on the kinetics of reappearance of activity (Fig. 3). Exposure of the I form to pH 11.5 for 10 s produced 11% of the potential activity within 10 s of the pH drop to pH 7.3, whereas a 5-min incubation at pH 11.5 gave 9% potential activity 10 s after the pH drop. The remainder of the activity reappeared slowly, at a rate which decreased with increasing delay time at pH 11.5. Again, unfolding was found to be fully reversible for incubations up to 5 min at pH 11.5.

Absorbance Changes during Refolding—Previous studies on the kinetics of absorbance changes during refolding were

![Fig. 1. The time course of the slow absorbance change after unfolding of pepsinogen at pH 11.5. Equal volumes of a 25-mg/ml solution of pepsinogen and bicarbonate buffer were mixed in a cuvette, and absorbance followed at 295 nm (○). The data were fitted to a first order curve, rate constant k = 0.0045 s⁻¹. Inset, an Arrhenius plot showing the variation of k with temperature.](http://www.jbc.org/)

![Fig. 2. Double jump experiments, measuring the reappearance of potential pepsin activity at pH 7.3, after jumping to pH 11.5 from neutrality. ○, activity at pH 1.8, as a function of time after a jump to pH 11.5. Other solutions were jumped to pH 11.5, held there for a variable delay time, and then the pH was rapidly dropped to pH 7.3. Activity was assayed at pH 1.8 as a function of time after the pH drop. Delay times were: V, 10 s; □, 30 s; Δ, 1 min; and ○, 5 min.](http://www.jbc.org/)
all performed after the protein was maintained at high pH for one convenient time period, i.e. 30 s. Absorbance changes on unfolding were thought to be complete, and irreversible denaturation was not detected (4). The present observations indicate the importance of studying the effect of varying the delay time at pH 11.5. Marked changes were found both in the size of the absorbance changes resolved in these experiments and in their time courses (Fig. 4). The size of the resolved change in molar absorptivity, estimated for each delay time and obtained by extrapolation of these curves to zero time of refolding was found to increase from \( \Delta E_0 = +1800 \) after 10 s at pH 11.5 to \( \Delta E_0 = +1450 \) after 5 min. Refolding was complete in all cases, as judged by recovery of potential pepsin activity. At early times the absorbance change followed an exponential time course, with rate constant \( k_1 = 0.02 \) s\(^{-1}\), but with increasing delay time after 30 s a slower reaction with rate constant \( k_2 = 0.004 \) s\(^{-1}\) became increasingly evident. The total resolved change in molar absorptivity was found to vary in parallel with the rapidly recovered fraction of the potential pepsin activity, estimated from Fig. 2, and to follow apparent first order kinetics with the delay time, with a rate constant, \( k_{app} \) of 0.025 s\(^{-1}\) (Fig. 5). Similar effects were seen at 30 and 35 °C, except that the rate constants were found to be functions of temperature. Over this limited range of temperature the enthalpies of activation for the three reactions were found to be \( \Delta H^\ddagger_1 = 2 \pm 1 \) kcal mol\(^{-1}\), \( \Delta H^\ddagger_2 = 12 \pm 1 \) kcal mol\(^{-1}\), and \( \Delta H^\ddagger_{app} = 26 \pm 2 \) kcal mol\(^{-1}\).

Similar experiments were performed to study the behavior of unfolded pepsinogen molecules (U) which originated from the inactive form (I). Protein solutions were incubated at pH 10 for 15 min and then jumped to pH 11.5. After a carefully measured interval under these conditions, the solution was neutralized and the absorbance changes accompanying refolding were measured (Fig. 6). The time course of the reaction was biphasic, again with rate constants \( k_1 = 0.02 \) s\(^{-1}\) and \( k_2 = 0.004 \) s\(^{-1}\). The estimated size of the total resolved change in molecular absorbptivity was a weak function of the delay time at pH 11.5, increasing from \( \Delta E_0 = +1450 \) after 10 s to +2000 after 5 min. Most of the change resulted from an increase in the contribution of the slower phase to the total change.
These studies show that the alkaline unfolding of swine pepsinogen is much more complex than was previously reported (3, 4). They indicate that the rapid unfolding previously detected above pH 10 by the ionization of tyrosine residues in the protein is followed by several other reactions. A further structural transition is indicated by a small slow change in absorbance whose rate is independent of pH or ionic strength but increases markedly with temperature. Refolding studies demonstrate that this is not due to irreversible denaturation of the protein, since no loss in thezymogen’s potential proteolytic activity could be detected after 5 min at pH 11.5, 25 °C, when this slow absorbance change was almost 70% complete (Fig. 1). However, the mechanism of refolding is found to change over the same time period. After 10 s at pH 11.5 over 80% of thezymogen is in a form which can be activated to pepsin immediately after returning to neutrality. Five minutes later, only 14% of the molecules show this property; in each instance, the remainder of the protein became activatable through a slow reaction (Fig. 2). At this pH and temperature, unfolding from the native form (N) is complete in 3 s (4). Similar changes in refolding properties could be measured by absorbance changes (Fig. 4), which measure burial of aromatic side chains in the course of folding. After short delay times at pH 11.5, only a small slow change in absorbance could be detected although this increased in size with the delay time. The time course of the absorbance changes on refolding also became more complex with increasing delay times at pH 11.5.

Similar observations with other proteins have been interpreted as indicating a transition in the unfolded molecule from a fast refolding form to one or more slowly refolding forms (9). There is very strong evidence that this transition results from cis-trans isomerizations of proline residues in the protein, away from the configurations found in the folded form. With increasing time under unfolding conditions, the rate of refolding becomes limited by reversal of these slow reactions at “essential” prolines, which must have the correct configuration for attainment of the native structure. Fig. 5 shows that this slow transition in unfolded pepsinogen can be approximated by a first order curve whose rate constant, 0.025 s⁻¹, and enthalpy of activation, 26 kcal mol⁻¹, fall in the range expected for this kind of reaction. Since pepsinogen contains 18 prolines (10), a number of such transitions might be anticipated. It seems likely that the double jump procedure detects primarily the fastest isomerization reaction(s) in the unfolded protein. The increased complexity of refolding kinetics after longer periods at pH 11.5 suggests the presence of slower reactions. The slow absorbance change shown in Fig. 1 is also similar to that expected for proline isomerization; its rate is independent of pH but the reaction is highly temperature dependent, ΔHf = 24 kcal mol⁻¹. At 25 °C, pH 11.5, it occurs six times more slowly than the reaction shown in Fig. 5. Garel (11, 12) has shown that proline isomerization reactions can be detected both in a hexapeptide and an unfolded protein by absorbance changes at adjacent nitrotyrosine residues. The sequence of pepsinogen is shown in two Tyr-Pro bonds (10). It is conceivable that the slow absorbance change is associated with the isomerization of one or other of these bonds.

After 5 min at pH 11.5, 95% of thezymogen is present in slowly folding forms. Yet the kinetically resolved change in molar absorptivity under refolding conditions (+1800) is much smaller than that expected from equilibrium unfolding measurements (+9000) (5). It has been argued elsewhere that this indicates the rapid formation of one or more partially folded intermediates during folding (4, 5). The data in Fig. 5 show that these intermediates must arise from the slowly folding forms of the protein. (As yet, folding after very short times at pH 11.5 is too fast for study of mechanism.) To accommodate these observations, one can write a general model for unfolding-refolding reactions.

\[ \begin{align*}
U_1 & = \text{U} = \text{U}_2 = \ldots = \text{N} \\
I & = I_2 = \ldots = I_3
\end{align*} \]

where N is the native protein, U₁ the initial unfolded form, and U₂, U₃, etc. are all slowly folding unfolded forms produced presumably by subsequent isomerization of one, two, or more essential proline residues. I₁, I₂, etc. are partially folded intermediates produced from U₁, U₂, etc. These intermediates must undergo proline isomerizations before they can fold fully to N.

How can the alkaline-inactivated (I) form of pepsinogen be accommodated into Scheme 2? In agreement with previous results (3-5), the I form unfolds much more quickly than the native protein. However, the present data show that the initial product of the reaction is changed. Immediately after unfolding, all of the protein is in a slowly refoldable form as judged by two criteria (Figs. 3 and 6). Extended exposure to pH 11.5 makes the refolding reaction both slower and more complex. The simplest way to explain these facts is to identify the inactive form (I) with a subpopulation of I₁, in which one proline is in the wrong configuration. Proline isomerization will destabilize the protein structure and, unlike a conformational change, its effects will persist after unfolding. Rapid unfolding would produce U₁ which refolds to N slowly and can further isomerize to U₂, U₃, etc.; the latter set of forms would refold even more slowly. Evidence for structural changes from such an unfolded form is seen in the continued presence of the small and slow absorbance change after rapid unfolding. The small increases in size of the resolved refolding absorbance change with longer delay times at pH 11.5 in the double jump experiments can be taken to indicate ensuing changes in the structure of the intermediates produced by pH drops (Fig. 6). The data also show that the refolding mechanisms of the unfolded forms produced from native (N) and inactive (I) forms of pepsinogen become more alike with increasing exposure to pH 11.5. This result would be anticipated from Scheme 2, as the unfolded forms undergo further isomerization to the same equilibrium distribution.

Scheme 2 implies that N and I₁ are reversibly connected. The refolding kinetics demonstrate the formation of N from I₁, showing that proline isomerization can occur during folding (the reason for the low ΔHf values of these reactions has been discussed by Nall et al. (13)), but it is generally thought that this reaction is initiated after unfolding. Since the inactivation and unfolding equilibria overlap at high pH, the possibility must be entertained that N is converted to I₁, through transiently formed U₁ and U₂. The possibility of such “unfolding” transitions between very similar forms of a protein was predicted some years ago by Lumry and Biltonen (14). However, Reeke et al. (15) have suggested that the conformational change accompanying the release of metal ions from concanavalin A includes the cis-trans isomerization of a peptide bond. Thus, such reactions may occur in folded proteins.

Earlier studies on unfolding of pepsinogen by urea did not show two phenomena predicted by Scheme 2, which were detected in these studies. All of the absorbance changes on unfolding followed a simple exponential time course. There was no sign of fast and slow phases (3). Double jump experiments failed to reveal a hidden transition after unfolding from fast to slow folding forms. Conversely, refolding experiments showed complex kinetics indicating the presence of intermediates. The temperature dependence of the rates of unfolding and refolding indicated that conversion between the inter-
The intermediates and the native protein involved proline isomerization (5). Similar results have been obtained with other proteins such as penicillinase (16) and the α-subunit of tryptophan synthetase (17, 18) and have also been interpreted to show stable obligatory intermediates in unfolding and refolding. The connection between these studies and the present ones can be made through the theoretical studies of Creighton (19).

The unfolding of a protein containing n trans prolines in its native state can be idealized as:

\[
\frac{k_u}{k_s} \frac{U_1}{U_0} = \frac{n k_c}{k_t} \frac{(n-1) k_u}{2 k_t} \frac{(n-2) k_u}{3 k_t} \frac{k_u}{n k_u} \rightarrow U_n
\]

Here, \(k_u\) and \(k_s\) are the rate constants of unfolding and refolding, and \(k_c\) and \(k_t\) are those for cis-trans isomerization, assuming all prolines to be identical. The statistical factors indicate the possibilities for isomerization between the different species, which are as in Scheme 2. Because of the large number of unfolded forms, mass action will weight the equilibrium toward unfolding by a factor of \((1 + k_u/k_t)^n\). Consequently, for a protein like pepsinogen, with 18 prolines (19), unfolding could be favored by a factor of 1.2\(^{18}\) to 1.6\(^{18}\), i.e. 27 to 4722. Thus, proline isomerization can drive unfolding to completion even when \(k_u/k_s\) is very small. The reaction will occur without the large transient population of \(U_0\) that would be necessary to see biphasic kinetics of unfolding and for successful double jump experiments (6). Furthermore, the product of unfolding will be a mixture of \(U_1\) to \(U_n\), all with incorrect proline residues. The intermediates detected during refolding will be trapped by these prolines and will not lie on the most direct route of folding. For large proline-rich proteins information on this direct route may only be obtained through double jump experiments which first unfold the proteins under the most extreme conditions, such that \(k_u \gg k_s\), thereby avoiding this trap.

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Intermediates in Pepsinogen Folding 693
The origin of the intermediates detected in the folding of swine pepsinogen.

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