Unfolding and Refolding of *Coprinus cinereus* Peroxidase at High pH, in Urea, and at High Temperature. Effect of Organic and Ionic Additives on These Processes[†]

Jeppe W. Tams and Karen G. Welinder*

Department of Protein Chemistry, Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 K, Copenhagen, Denmark

Received December 28, 1995; Revised Manuscript Received March 22, 1996[⊗]

ABSTRACT: The unfolding and refolding rates of the heme- and Ca^{2+}-containing *Coprinus cinereus* peroxidase (CIP) have been measured at pH 12.1, in 4 M urea, and at 61.2 °C. The change in peroxidase activity paralleled the change in the Soret band absorbance of the heme group. The unfolding rate constant (k_u), was determined independently in thermolysin digestion and EDTA experiments at 59.4 °C. Both gave k_u values of 1.5 ms^{-1}, and also showed that the presence of 4 mM EDTA made CIP unfolding practically irreversible. Unfolding and refolding rates could therefore be determined under identical conditions of denaturation having either EDTA or Ca^{2+} in excess. The refolding rates at high pH and in 4 M urea were measured by adding Ca^{2+} to the unfolded CIP, whereas refolding at 61.2 °C was evaluated by comparing the unfolding carried out under reversible (excess of Ca^{2+}) and irreversible conditions (excess EDTA). The activation energies for the unfolding at 61.2 °C are approximately $\Delta G^{\ddagger}_u$ 100, $T\Delta S^{\ddagger}_u$ 200, and $\Delta H^{\ddagger}_u$ 300 kJ/mol. Five different additives, glycerol, EtOH, Na_2SO_4, guanidinium chloride (GdmCl), and NaCl, all at 100 mM, were used as probes to evaluate the mechanism of base, urea, and heat on unfolding and refolding. Salts destabilized CIP at high pH, primarily by enhancing the unfolding rate but also by decreasing the refolding rate. Glycerol had the reverse effects and thus stabilized CIP at high pH. The unfolding rate in urea was only slightly affected by the additives, with the exception of GdmCl which enhanced the unfolding rate. The refolding rate was decreased in urea by EtOH and GdmCl, in contrast to glycerol and Na_2SO_4 which increased the refolding rate. At high temperature the unfolding was affected only slightly by the additives, except for GdmCl, and to a lesser extent NaCl, which enhanced the unfolding rate. The refolding rates were greatly decreased by Na_2SO_4, EtOH, and GdmCl, whereas glycerol and NaCl enhanced the process. It appears that 100 mM NaCl functions as a catalyst for the temperature-induced process, enhancing both the unfolding and refolding rates. The results indicate that the mechanisms of CIP unfolding and refolding are similar in urea and at high temperature but different at high pH.

Preservation of the native conformation of a protein is essential for biological activity. The native conformation, however, is stabilized only by 20−40 kJ mol^{-1} under normal physiological conditions, and protein stability in technical applications is therefore of major concern. Factors influencing the conformational stability have been studied most extensively in small globular proteins under reversible conditions (Tanford, 1970; Privalov, 1979). Many proteins cannot refold *in vitro* due to the lack of chaperonins, protein disulfide isomerase, peptidylprolyl cis−trans isomerase, proper reducing potential, and post translational modification, such as the removal of a propeptide (Creighton, 1992). Proteins which can refold are exposed to irreversible loss of activity. The major pathway for irreversible loss of protein activity goes through the unfolded state N ⇌ U → I (N, native; U, unfolded; I, inactive) (Volkin & Klibanov, 1989), as the unfolded state is more accessible to the environment than the native state. Therefore, the unfolding rate constant is the most important measure of protein stability for all practical purposes. The U→I process may be caused by proteolytic digestion (Imoto et al., 1987), autoaggregation or aggregation to other surfaces, loss of prosthetic group, cis−trans isomerization of certain proline residues (Levitt, 1981; Schmid et al., 1993), or chemical modifications (Ahern & Klibanov, 1985). Modifications, such as deamidation of asparagine side chains, in particular, and β-elimination of disulfide bridges, will have a significant effect on the refolding capability of a protein if amino acid residues of the protein interior are modified. In contrast, modification of surface amino acids has only a minor effect.

Protein folding research has recently expanded further due to the need for folding of proteins from inclusion bodies produced by over expression in *Escherichia coli.* In many cases, folding has been achieved by manipulation with additives (Cleland & Wang, 1990; Wetlaufer & Xie, 1995). The efficiency of protein folding is largely determined by the rates of folding, unfolding, and aggregation. Therefore, it is important to know the influence of additives on these rate constants to obtain high folding yields.

The known effect of additives on protein stability is mainly based on high concentrations of additive (Timasheff, 1993). Here we have determined the effects of additives at 100 mM concentrations. Additives exert their effect through two different mechanisms. (i) At high additive concentration the properties of water are changed. (ii) At low concentration

[†] This research was supported by the Danish Technology Council Grant 133/001-9000090.

* Author to whom correspondence should be addressed. FAX: +45 35 32 20 75. E-mail: welinder@biobase.dk.

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1996.

S0006-2960(95)03067-4 CCC: $12.00

specific binding to the protein is predominant. The same phenomenon is the basis for salting in and salting out of proteins (Timasheff & Arakawa, 1988). Using low concentrations of additives, information is obtained on the relative exposure of hydrophobic and hydrophilic surfaces in the native, transition, and unfolded states.

In this paper we have studied the unfolding and refolding rate constants as a function of three types of denaturation conditions, i.e., pH, urea, and temperature denaturation. In addition, the effects of five representative additives were studied: Na_2SO_4, an ionic stabilizer, and a cosmotroph salt in the Hofmeister series; GdmCl, a strong ionic denaturant mainly acting in competitive hydrogen-bond formation; glycerol, a non-ionic stabilizer exerting a solvophobic effect; EtOH, a non-ionic denaturant with a preference for hydrophobic surfaces above 20 °C (Brandts, 1969); and finally NaCl which is neither caotrophic nor cosmotrophic in the Hofmeister series (rating the power of water-ordering properties of the ions).

In a previous study on the physico-chemical effects of glycans on the activity, solubility, and stability of horseradish peroxidase (J. W. Tams and K. G. Welinder, unpublished; Welinder & Tams, 1995; Tams & Welinder, 1995), we found that peroxidases are ideally suited for the study of kinetic stability of proteins for two reasons. (i) Heme can serve as a built-in spectroscope probe of the N and U folding states. (ii) The two structural calcium ions present in secretory plant and fungal peroxidases can be trapped efficiently by EDTA during the unfolding, hence permitting a very precise measurement of the unfolding rate constant (k_u) independently of the folding rate constant (k_f). In the present study we use the well characterized recombinant *Coprinus cinereus* peroxidase (CIP) expressed in *Aspergillus oryzae* (*Arthromyces ramosus* peroxidase (ARP) is an alternative name for CIP) (Kjalke et al., 1992; Baunsgaard et al., 1993; Limongi et al., 1994; Petersen et al., 1994; Kunishima et al., 1994; Fukuyama et al., 1995; Veitch et al., 1994; Smulevich et al., 1994). CIP has M_r 38 000 and p*I* near 3.5. This peroxidase and designed mutants of increased stability show great promise for biotechnological applications (Pedersen & Carlsen, 1994; Cherry et al., 1995). Recombinant CIP has become our preferred model system for protein and glycoprotein stability studies, as highly homogeneous wild-type and a number of mutant CIP enzymes have been obtained in quantity (Welinder & Andersen, 1993). CIP is stable (G. Smulevich et al., submitted) and active from pH 4−12 (Andersen et al., 1991b; A. K. Abelskov et al., submitted). One of the conclusions of the present study is the importance of electrostatic interactions for the stability of this class of heme-containing peroxidases. We also discuss the likely mechanisms of unfolding and refolding of the heme and calcium ion containing plant and fungal peroxidases.

EXPERIMENTAL PROCEDURES

CIP and ApoCIP. Recombinant CIP was expressed in transformed *A. oryzae* (Dalbøge et al., 1992; Vind, 1994). The fermentation broth was filtered, and CIP was precipitated with ammonium sulfate and purified to homogeneity (RZ 3.0) in three chromatographic steps: (i) anionic exchange chromatography was performed on a column of HiLoad Q-Sepharose in Bis-Tris buffer, pH 6.0; (ii) concanavalin A-Sepharose affinity chromatography; (iii) rechromatography on the Q-Sepharose column in sodium acetate buffer, pH 4.7 (Limongi et al., 1995). SDS−PAGE, absorption spectroscopy, and RZ-values were used for homogeneity criteria. ApoCIP was prepared by removing heme at pH 2 by extraction with 2-butanone (Teale, 1959).

Unfolding/Refolding Kinetic Studies. The pH-induced unfolding of CIP (0.20 mg/mL) was performed in a solution of freshly prepared 12 mM NaOH, 4 mM tetrasodium EDTA, 8 μM heme, pH 12.1 at 16.3 °C, and initiated by the addition of CIP at $t = 0$ s. The absorbance at 410 nm was recorded for 0−600 s. After 800 s under unfolding conditions, refolding was initiated by adding a solution of 420 mM $CaCl_2$ in 12 mM NaOH. The final conditions for folding were 0.19 mg of CIP/mL, 12 mM NaOH, 19 mM $CaCl_2$, 3.8 mM tetrasodium EDTA, 7.6 μM heme, pH 12.1, 16.3 °C. Again the absorbance at 410 nm was recorded for 600 s.

Unfolding of CIP (0.20 mg/mL) by urea was performed at 37.3 °C using 6 M urea, 4 mM disodium EDTA, 31 mM TrisHCl, 2.7 μM heme, pH 7.9. After 800 s refolding was initiated by dilution with 40 mM $CaCl_2$ to a final CIP concentration of 0.13 mg/mL in 4 M urea, 13 mM $CaCl_2$, 2.6 mM disodium EDTA, 21 mM TrisHCl, 1.8 μM heme, pH 7.9 at 37.3 °C. The absorbance at 405 nm was recorded for 600 s. Similar unfolding studies were performed in 4 M urea and recorded for 600 s.

Irreversible temperature unfolding was performed using 0.20 mg of CIP/mL in 40 mM TrisHCl, 4 mM disodium EDTA, pH 7.6 at 56.0 °C and pH 7.4 at 66.4 °C. The absorbance at 405 nm was recorded for 1200 s. Reversible temperature unfolding was performed using similar conditions, but excluding EDTA and including 8 mM $CaCl_2$, 2.7 μM heme.

Irreversible temperature induced unfolding was also studied by proteolysis. Thermolysin digestions were performed by incubation of 0.20 mg of CIP/mL, 40 mM TrisHCl pH 8.0, 2.6 μM heme, 20 mM $CaCl_2$, and 0.02−0.10 mg of thermolysin/mL at 59.4 °C. The reactions were monitored at 405 nm for 20 min.

Similar experiments were performed in the presence of 100 mM of the various additives in the unfolding and refolding mixtures. All experiments were performed in duplicate, and the series of experiments as presented in Tables 1, 2, and 3 were carried out on the same day using the same stock solutions of reagents. Hence, the relative effects of additives were highly reproducible, whereas the day to day deviations were greater.

Peroxidase Activity. Samples were analyzed for peroxidase activity using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate (Arnao et al., 1990). After incubation samples were diluted immediately in 40 mM TrisHCl, 4 mM EDTA, pH 8.0 at room temperature to arrest further uncontrolled reactions, such as refolding, which would take place in the absence of EDTA, or unfolding, which is negligible at pH 8 at room temperature. The assay conditions were 0.18 nM CIP, 100 μM H_2O_2, 1 mM ABTS in 50 mM sodium phosphate buffer, pH 6.3, and the increase in absorbance at 414 nm due to ABTS radical formation was recorded.

Spectroscopic Studies and Analyses of Transition Curves. Spectral changes were recorded on a Beckman DU 70 spectrophotometer. The cuvette was thermostated by means of a circulating water bath. Reactions were monitored as the decrease in Soret band absorbance (*A*) at 405 nm at

neutral pH and at 410 nm at pH 12.1. Data were collected at a sampling rate of 1 s^{-1} and transferred to a spread sheet. ΔA was plotted as a function of time t.

$$\Delta A = (A_t - A_\infty)/(A_0 - A_\infty) \quad (1)$$

A_t is the absorbance at time t during unfolding or folding, and A_∞ is the absorbance at the end of the reaction (Schmid, 1992). At neutral pH at 405 nm, $\epsilon = 109$ mM^{-1} cm^{-1} for native CIP (Andersen et al., 1991a) and 40 mM^{-1} cm^{-1} for unfolded CIP, determined from the endpoint of the reaction and corresponding to free heme. The same values were used at high pH at 410 nm. The experimental data were fitted to first-order expressions

$$\Delta A = \exp(-t/\tau) \quad (2)$$

$$\Delta A = P_1 \exp(-t/\tau_1) + P_2 \exp(-t/\tau_2) \quad (3)$$

where τ is the time constant for a monophasic reaction (eq 2) or for a biphasic reaction (eq 3). P_1 and P_2 indicate the fractions reacting with time constants τ_1 and τ_2, respectively. For any set of experimental conditions the time constant and the rates of unfolding k_u and folding k_f are related by

$$\tau = 1/(k_u + k_f) \quad (4)$$

When folding is arrested by EDTA and $k_f = 0$, then

$$\tau = 1/k_u \quad (5)$$

The change in transition free energy of unfolding in the presence of an additive is

$$\Delta\Delta G^{\ddagger}_u = RT \ln(k_{u,none}/k_{u,additive}) = RT \ln(\tau_{additive}/\tau_{none}) \quad (6)$$

RESULTS

The experimental conditions for pH-, urea-, or temperature-induced unfolding and refolding of CIP were adjusted in such a way that the reactions could be followed on a convenient time scale and that the possible interference of cis−trans isomerization of proline residues of unfolded protein would be reduced. The unfolding reaction could be performed under irreversible conditions by trapping the two structural Ca^{2+} ions of CIP by EDTA (substantiated below). The corresponding refolding process could, in most cases, be performed under nearly identical conditions by adding an excess of Ca^{2+} dissolved in a small volume of the unfolding solution. The change in the absorption spectrum during the reactions showed an isosbestic point. This signifies that heme is either bound, as in the native enzyme, or released completely to free heme and that a two-state process is taking place.

Unfolding and Refolding of CIP at High pH. The pH-dependent unfolding and refolding of CIP were performed in 12 mM NaOH, pH 12.1 at 16.3 °C. Figure 1A shows that tracing the reactions either by residual peroxidase activity or by change in Soret absorbance gave identical results within experimental errors. Analyzing the change in absorbance at 410 nm enabled two distinct unfolding phases to be observed with time constants of 36 and 569 s, respectively. The fast reaction accounted for 14% and the slow reaction accounted for 86% of the total change in absorbance. The two phases might be due to the presence of different forms of CIP (G. Smulevich et al., submitted) which vary with respect to stability. Refolding was initiated after 800 s of unfolding by addition of excess Ca^{2+}. Two phases were also observed in this case: a fast reaction with a time constant of 27 s accounting for 73% of the change in absorbance, and a slow reaction with a time constant of 180 s accounting for 27%. These two phases could reflect the presence of different unfolded forms of CIP.

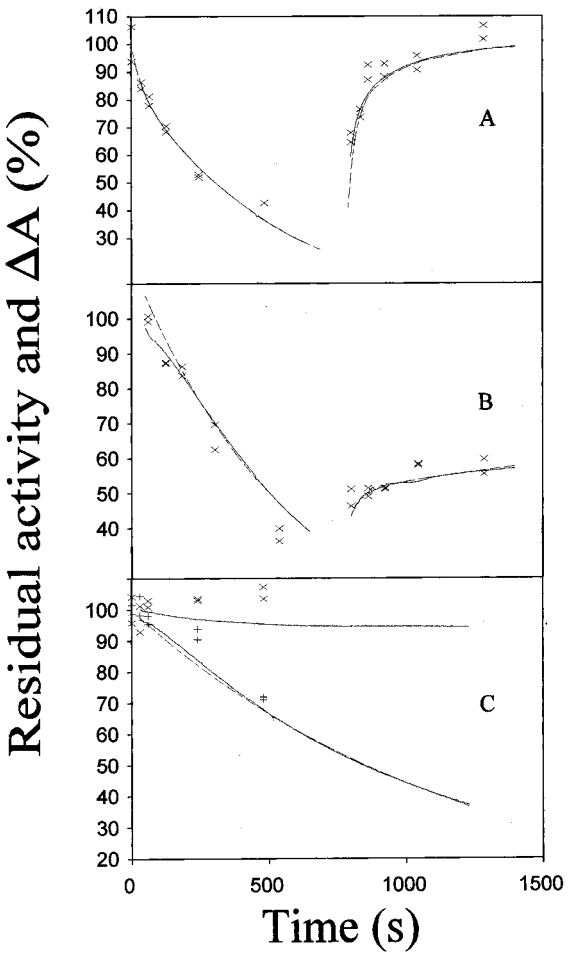

FIGURE 1: Unfolding and refolding of CIP, (A) at high pH (pH 12.1, 16.3 °C; ΔA at 410 nm), (B) in urea (6 M urea for unfolding, 4 M for refolding, at pH 7.9, 37.3 °C; ΔA at 405 nm), and (C) at high temperature (59.4 °C, pH 7.5; ΔA at 405 nm). In C, irreversible unfolding (lower curve) was performed in the presence of EDTA, and reversible unfolding (upper curve) was performed in the presence of Ca^{2+}. Changes in peroxidase activity (× and +) and in Soret absorbance (solid line) are coincident in all cases. The dashed lines are theoretical curves fitted to the observed changes in absorbance (%): $\Delta A = \exp(-t/\tau) \times 100$ for monophasic unfolding, $\Delta A = P_1 \exp(-t/\tau_1) + P_2 \exp(-t/\tau_2)$ for biphasic unfolding, and $\Delta A = 100 - P_1 \exp(-t/\tau_1) - P_2 \exp(-t/\tau_2)$ for biphasic folding (see Experimental Procedures).

Unfolding and Refolding of CIP in Urea. CIP was unfolded in 6 M urea, pH 7.9 at 37.3 °C in the presence of 4 mM EDTA. The change in absorbance at 405 nm showed an initial lag period followed by an apparent first-order decay (Figure 1B). The calculated urea unfolding time constant was 596 s (data points from 500 to 600 s were used for fitting). After unfolding for 800 s refolding was initiated by dilution to 4 M urea with 40 mM $CaCl_2$. Here two phases were observed, a fast reaction with a time constant of 36 s accounting for 27% of the change in absorbance, followed by a slow reaction with a time constant of 4210 s and accounting for 73%.

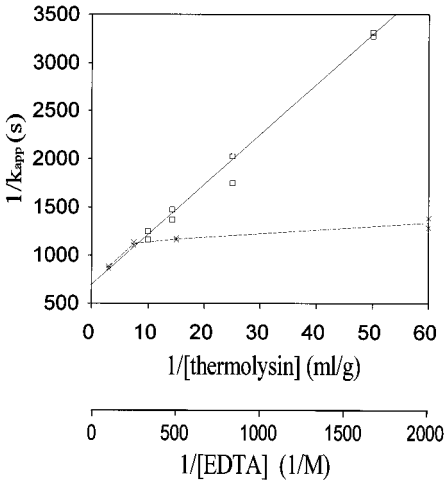

FIGURE 2: Irreversible unfolding of CIP at 59.4 °C in the presence of thermolysin (□) and EDTA (×). Thermolysin digestions were performed using 0.20 mg of CIP/mL, 40 mM TrisHCl, 20 mM $CaCl_2$, 2.6 μM heme, pH 7.5, and 0.02−0.10 mg of thermolysin/mL. Ca^{2+}-trapping experiments were performed using 0.20 mg of CIP/mL, 40 mM TrisHCl, pH 7.5, and 0.5−10 mM EDTA. The k_{app} was determined by fitting the change in the Soret absorbance at 405 nm. Similar rate constants of 1.5 ms^{-1} corresponding to time constants of 700 s were obtained from the intercepts.

Temperature Unfolding of CIP under Irreversible and Reversible Conditions. Irreversible temperature-induced unfolding of CIP was performed at 59.4 °C in 4 mM EDTA, 40 mM TrisHCl, pH 7.5. A lag period was followed by an apparent first-order decay (data points from 400 to 1200 s, were used for fitting) with a time constant 1200 s (Figure 1C). Refolding, attempted by lowering the temperature, was not successful due to precipitation. A qualitative estimate of the refolding rate was instead obtained by following the unfolding in the presence of excess Ca^{2+} and heme. The reversible unfolding experiment at 59.4 °C, 8 mM $CaCl_2$, 40 mM TrisHCl, pH 7.5, showed very little unfolding, i.e., the refolding rate was much greater than the unfolding rate under these conditions.

Influence of Thermolysin Digestion and EDTA on Refolding. It has been shown by Imoto et al. (1986) that the unfolding rate constant for a protein can be determined accurately in a protease digestion experiment. The basis for this is the sequence of reactions N ⇋ U ⟞ peptides, where the native protein N usually is resistant to protease digestion, whereas the digestion of the unfolded form U shows saturation kinetics using increasing concentrations of protease. Native CIP fulfills this requirement and was resistant to thermolysin for 20 min at 30 °C, at conditions where all apoCIP was degraded (results not shown). Thermolysin digestions were performed in the presence of Ca^{2+} and heme such that only the irreversible step of proteolytic removal of U was affected. Figure 2 shows the results of thermolysin digestions of CIP at 59.4 °C as a double-reciprocal plot of the apparent unfolding rate constant versus the concentration of thermolysin. k_u was found to 1.5 ms^{-1} from the intercept at the *y* axis. Figure 2 also shows a double-reciprocal plot of the apparent unfolding rate constant and [EDTA] giving the same extrapolated k_u value. These results support that 4 mM of EDTA used throughout this work made the N to U process for CIP irreversible for all practical purposes, although EDTA and Ca^{2+} are in equilibrium with their complex. At low [EDTA] the unfolding of CIP was slightly reversible due to the low inherent [Ca^{2+}] resulting from CIP unfolding.

Table 1: Effect of Additives on the Stability of CIP at pH 12.1, 16.3 °C[a]

additive (100 mM)	unfolding τ_u (s)	$\Delta\Delta G^{\ddagger}_u$ (kJ/mol)	folding τ_f (s)
none	540 ± 30	0	27 ± 1
glycerol	770 ± 10	0.9 ± 0.1	20 ± 2
EtOH	540 ± 70	0.0 ± 0.3	29 ± 1
Na_2SO_4	370 ± 10	−0.9 ± 0.1	27 ± 1
GdmCl	110 ± 10	−3.8 ± 0.1	44 ± 8
NaCl	330 ± 30	−1.2 ± 0.2	32 ± 3

[a] The time constants for the major phase of the biphasic reactions are shown (compare Figure 1A).

Effect of 100 mM of Additives. The results are reported in Tables 1−3 for base, urea, and temperature experiments, respectively. The effects of the various additives were either to increase or decrease the time constants, although the overall reaction followed the same pattern as the reference experiment with no additive regarding lag and monophasic or biphasic kinetics (see Figure 1A−C). Only the time constant for the major component of a reaction is reported. The effects of additives on the minor phases, when present, were not qualitatively different from the corresponding major phases. Comparison of the data reported in the previous paragraphs for experiments with no additive, and the similar reference experiment marked "none" of Tables 1−3, shows slight differences which indicate the day to day experimental variation. In contrast, all the results in a table were carried out with the same reagents on the same day (see Experimental Procedures).

Effect of Additives at High pH. Table 1 shows a summary of the effects of two organic and three ionic additives on the time constants τ_u meassured for irreversible unfolding and the corresponding stabilizing (positive) or destabilizing (negative) effects, expressed as the change in unfolding free energy $\Delta\Delta G^{\ddagger}_u$ relative to the experiment with no additive. The time constants τ_f meassured for reversible folding are also shown. Glycerol increased the unfolding time constant corresponding to $\Delta\Delta G^{\ddagger}_u = 0.9$ kJ/mol and, at the same time, decreased the refolding time constant. Thus glycerol is a true stabilizer of CIP at pH 12.1 whether the process is reversible (N ⇋ U) or irreversible (N ⟞ U). EtOH had no detectable effect neither on the unfolding, nor on the refolding time constants. In contrast, the ionic additives all decreased the stability of CIP markedly at high pH, as all reduced the unfolding time constants, and NaCl and GdmCl moreover increased the refolding time constant. Thus the effects of these three salts follow the Hofmeister series. All are denaturants, however.

Effect of Additives in Urea. Table 2 summarizes the results obtained in urea. There was no effect on the unfolding time constant adding glycerol, Na_2SO_4 or NaCl to 6 M urea, whereas EtOH and GdmCl showed decreased time constants, or faster unfolding. The time constants for the unfolding of CIP in 4 M urea in the presence of glycerol, Na_2SO_4, or NaCl were therefore also assumed to be unchanged. EtOH and GdmCl in 4 M urea showed the same tendencies as in 6 M urea, although this was less obvious for EtOH. The slow, but dominant refolding phase of CIP in 4 M urea had a time constant of 4500 ± 300 s. Glycerol and Na_2SO_4 decreased the refolding time constants to 3300

Table 2: Effect of Additives on the Stability of CIP in Urea at 37.3 °C, pH 7.9[a]

additive (100 mM)	6 M urea τ_u (s)	4 M urea τ_u (s)	$\Delta\Delta G^{\ddagger}_u$ (kJ/mol)	τ_f (s)
none	595 ± 4	6500 ± 100		4500 ± 300
glycerol	599 ± 4	*b*	+0.8 ± 0.4	3300 ± 500
EtOH	398 ± 9	6200 ± 100	−0.12 ± 0.04	7000 ± 400
Na_2SO_4	602[e]	*b*	+1.2 ± 0.1	2800 ± 100
GdmCl	169 ± 8	2600 ± 100	−2.4 ± 0.1	*c*
NaCl	599 ± 4	*b*	∼0	*d*

[a] The time constants for unfolding are for a monophasic reaction after an initial lag period. The time constants for folding are the major phase of a biphasic reaction after a lag period (compare Figure 1B). [b] Assumed to be unchanged by the additive as seen in 6 M urea. [c] No refolding is seen, thus $\tau_u > 10\,000$ s. [d] The data could not be fitted due to major oscillations, presumably resulting from poor mixing. The refolded fraction after 1500 s was approximately the same as without additive. [e] One experiment only.

Table 3: Effect of Additives on the Unfolding of CIP at 61.2 °C, pH 7.4[a]

additive (100 mM)	irreversible[b] τ_u (s)	$\Delta\Delta G^{\ddagger}_u$ (kJ/mol)	reversible[c] τ_u (s)
none	660 ± 30		4800 ± 100
glycerol	660 ± 10	0 ± 0.1	11000 ± 2000
EtOH	610 ± 40	−0.2 ± 0.4	3200 ± 100
Na_2SO_4	610 ± 50	−0.2 ± 0.4	3800 ± 100
GdmCl	240 ± 10	−2.8 ± 0.1	820 ± 20
NaCl	520 ± 10	−0.6 ± 0.1	6800 ± 100

[a] The time constants are derived for a monophasic reaction after an initial lag period (compare Figure 1C). [b] In the presence of 4 mM EDTA. [c] In the presence of 8 mM $CaCl_2$, 2.7 μM heme.

± 500 s and 2800 ± 100 s, respectively. Therefore, these two agents have no effect on irreversible unfolding but will stabilize under reversible or equilibrium conditions. In contrast, GdmCl is a strong enhancer and EtOH a moderate enhancer of both the reversible and irreversible processes in urea.

Effect of Additives at High Temperature. At 61.2 °C the unfolding of CIP was studied under both irreversible (EDTA present) and reversible (Ca^{2+} present) conditions. The results are remarkably different in these two cases as shown in Table 3. Glycerol has no effect on the irreversible unfolding reaction, but greatly stabilizes CIP under reversible conditions, indicating that glycerol increases the rate of refolding significantly. EtOH and Na_2SO_4 both showed insignificant effects under irreversible conditions and minor destabilization under reversible conditions, indicating a reduction of the refolding rate. As expected GdmCl showed marked destabilization of CIP in both reactions. NaCl destabilized CIP moderately in the irreversible experiment but showed a marked increase of the time constant for reversible unfolding. Hence NaCl catalyzed unfolding as well as refolding. It must be noted that the "reversible" unfolding processes could not be fitted to a simple N ⇌ U mechanism, indicating that N ⇌ U_{fast} ⇌ U_{slow} or that more complex processes of irreversible chemical modification took place.

Stability of CIP. The unfolding rate constant k_u for CIP unfolding was determined at temperatures from 37 to 68 °C in the presence of EDTA. Plotting ln k_u versus temperature showed a linear relationship (Figure 3). The $t_{1/2}$ values (time for the unfolding of half of CIP) were 1 s at 80 °C, 1 min at 67 °C, 1 h at 55 °C, 1 day at 45 °C, 1 month at 34 °C, and 1 year at 27 °C.

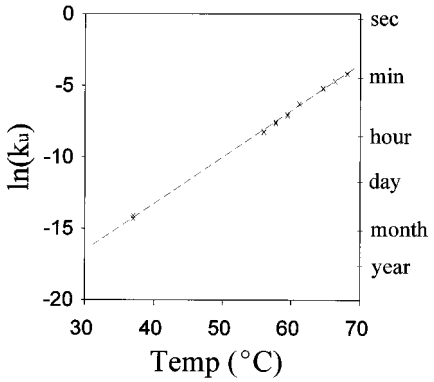

FIGURE 3: Kinetic stability of CIP in 4 mM EDTA, 40 mM TrisHCl as a function of temperature. At 37 °C the pH was 7.9. At 56−68 °C the pH was 7.6−7.4. The half-lives of CIP, $t_{1/2}$ (right-hand scale), stored under these conditions of irreversible unfolding were approximately 1 min at 67 °C and 2 weeks at 37 °C.

Table 4: Activation Free Energy Calculated for Diffeent Conditions of Unfolding

conditions	$\Delta G^{\ddagger}_u$ (kJ/mol)
37.3 °C, pH 7.9	112
4 M urea, 37.3 °C, pH 7.9	99
16.3 °C, *pH 12.1*	86
61.2 °C, pH 7.5	100

Plotting ln k_u versus the reciprocal temperature (Arrhenius plot) displayed a linear relationship (not shown). The gradient is equal to $-E_a/R$, where E_a is the activation energy and R is the gas constant. A value of $E_a = 315$ kJ/mol was obtained. The activation free energy was calculated according to the Eyring assumption (Finn et al., 1993) $\Delta G^{\ddagger}_u = -RT \ln(k_u h/(k_b T)) = 100$ kJ/mol at 61.2 °C (h is the Planck constant, k_b is the Boltzmann constant). As the activation enthalpy $\Delta H^{\ddagger}_u \approx E_a$, the contribution from the activation entropy $T\Delta S^{\ddagger}_u = \Delta H^{\ddagger}_u - \Delta G^{\ddagger}_u \approx 200$ kJ/mol at 61.2 °C, is substantial. The activation free energy $\Delta G^{\ddagger}_u$ for the major phase of unfolding at the different conditions of denaturation conditions was calculated from the Eyring assumption (Table 4). Relative to the mild conditions at 37 °C, pH 7.9, $\Delta G^{\ddagger}_u$ at pH 12.1 was decreased by 26 kJ/mol, in 4 M urea by 13 kJ/mol, and at 61.2 °C by 12 kJ/mol. It is also noteworthy that $\Delta\Delta G^{\ddagger}_u$ for 100 mM GdmCl was very high and accounted for approximately 20% of the total change, despite the assumed competition with urea.

DISCUSSION

Peroxidase Model. The fungal *C. cinereus* peroxidase, CIP, contains two structural Ca^{2+} ions and an Fe^{3+} heme group attached to the protein moiety by a single histidine ligand (Kunishima et al., 1994; Petersen et al., 1994). The heme group is essential for the peroxidase activity. The heme can be easily extracted at acid pH, and the resulting apo-peroxidase can be reconstituted with fresh heme at pH 7−9, preferably in the presence of excess Ca^{2+} (data not shown). Here we show that heme can serve as a convenient probe for the conformational stability of CIP. The change in peroxidase activity was found to parallel the change in Soret band absorbance (Figure 1). We also show that EDTA can trap structural Ca^{2+} released upon unfolding of CIP and thereby prevent refolding of CIP, giving $1/\tau \approx k_u$. The folding rate constant k_f can then be determined from $1/\tau \approx$

$k_u + k_f$ under the same denaturing conditions by adding an excess of Ca^{2+} relative to EDTA and apo-CIP.

High pH. The unfolding/refolding experiments at pH 12.1 and 16.3 °C showed a greatly decreased stability of CIP in the presence of salts. Even Na_2SO_4, which is generally regarded as a protein stabilizer, increased the unfolding rate constant indicating specific interaction with the protein. Thus the net denaturation effect on CIP at high pH cannot be due to negative charge repulsion at the surface but must be a result of disruption of salt bridges or of deprotonization of buried groups such as tyrosine. The increase in unfolding activation energy in the presence of 100 mM glycerol is in accord with the exclusion of glycerol from hydrophobic surfaces and that the hydrophobic surface increases N < transition state < U. The lack of a detectable effect of 100 mM EtOH is consistent with the observation that a low concentration of EtOH gives no marked binding to hydrophobic surfaces at room temperature (Brandts, 1969) and thus no preference for the unfolded state. The effect of additives at pH 12.1 was generally more pronounced for unfolding than refolding. The changes induced by additives showed that the unfolding was affected up to 3-fold more than the refolding reaction. This indicates that the transition state at pH 12.1 resembles U more than N and that the U state at pH 12.1 is a compact denatured state.

Urea. EtOH had a small effect on the unfolding rate in 4 M urea at 37.3 °C whereas no effect was seen for glycerol, Na_2SO_4, and NaCl, suggesting that there is no change in hydrophobicity of the surface of the transition state as compared to the N state and no specific ionic binding to transition state. Only GdmCl had a considerable effect, despite a presumed competition for the same hydrogen bonds as urea. In contrast, the additives had large effects on the refolding rates in 4 M urea. The U state is presumably considerably more hydrophobic than the transition state, as increases in the refolding rates were observed in the presence of 100 mM glycerol and Na_2SO_4, whereas EtOH decreased the refolding rate to the same extent. The small effect of additives on the unfolding and the large effect on the refolding rates suggest that the transition state is closer in structure to N than to U in urea, in contrast to the results at pH 12.1.

Temperature. The unfolding reaction at 61.2 °C was only marginally affected by additives under irreversible conditions, except for GdmCl. However the reversible unfolding, which represents a competition between the unfolding and refolding reactions and some unidentified irreversible process, was significantly affected by all additives (Table 3). Hence the transition state must be very different from U, and more similar to N. As the additives are non-covalent structural perturbants they are expected to only influence the conformational changes, not possible chemical modifications of the U state. The effects of 100 mM NaCl were special at high temperature. At irreversible unfolding NaCl acted as a denaturant indicating specific ionic binding to the transition state, whereas at largely reversible conditions, it showed a stabilizing effect. Hence it appears that 100 mM NaCl functions as a catalyst for the heat-induced N ⇌ U process.

In general, the equilibrium constant for the N ⇌ U process is informative only for fully reversible processes. For most practical applications of proteins, the unfolding rate constant, and thus the N ⇌ transition state equilibrium, is the most important parameter for preservation of the native conformation. The activation energies, $\Delta G^{\ddagger}_u$, $T\Delta S^{\ddagger}_u$, and $\Delta H^{\ddagger}_u$ at 61.2 °C were approximately 100, 200, and 300 kJ/mol, respectively.

GdmCl. The decrease in $\Delta\Delta G^{\ddagger}_u$ by 100 mM GdmCl is surprisingly similar for all the denaturing conditions (Tables 1−3), especially if the salt effect of the Cl^- is compensated for by subtracting the NaCl effect. Then $\Delta\Delta G^{\ddagger}_u$ becomes −2.6 kJ/mol at pH 12.1, −2.4 kJ/mol in 4 M urea, and −2.2 kJ/mol for temperature denaturation. This could indicate the same degree of backbone exposure and similar compactness of transition state structures in the presence of GdmCl.

Multiphasic Behavior. The unfolding and refolding of CIP were not simple N ⇌ U processes at all conditions. At high pH and in urea biphasic behavior was observed for both the unfolding and refolding, and the reversible unfolding at 61.2 °C could not be fitted to a two-state process. Despite the relatively short time of unfolding (800 s), the process consisted of at least $N \rightleftharpoons U_{fast} \rightleftharpoons U_{slow}$. The slow refolding phase could be a cis−trans isomerization of some proline residues, for which the time constant is 10−100 s (Schmid et al., 1993). However, a recent study of proline mutants of a *trp* apo-repressor showed that a slow phase still occurred in the folding of the des-Pro mutant (Mann et al., 1995).

Peroxidase As a Model for Protein Unfolding/Refolding. The studies reported in this paper address the questions of the mechanisms of protein denaturation by base, urea, and heat, and the effects of five selected additives on the unfolding and refolding using CIP as a model. The results refer to the well-known macroscopic two state reaction N ⇌ U (native ⇌ unfolded or denatured). For CIP we can write

$$[\text{CIP,heme,}2Ca^{2+}]_N \rightleftharpoons CIP_U + \text{heme} + 2Ca^{2+} \quad (7)$$

The complex shown in parentheses indicates native CIP, and unfolded forms of polypeptide CIP are indicated by CIP_U. In accord with this reaction scheme and the present results, the absorbance change at the Soret maximum for native CIP and the enzymic activity of CIP are coincident and will depend either on heme removal from CIP or rearrangement around the heme. The two-state reaction was supported by the presence of an isosbestic point for the absorption spectra of native and unfolded CIP, at 391 nm at pH 12.1 experiments, at 378 nm in urea, and at 367 nm in the heat experiments. Unfolded CIP had the spectral characteristics of free heme. However, stopped-flow experiments are likely to detect short-lived intermediates, as is well-known from other protein folding studies. Various evidence (see below) in fact suggests that the unfolding pathway for CIP may be described by reaction 8, whereas the folding is best described by reaction 9.

$$[\text{CIP,heme,}2Ca^{2+}]_N \rightleftharpoons [CIP_{U'}\text{,heme}] + 2Ca^{2+} \rightleftharpoons CIP_U + \text{heme} \quad (8)$$

$$[\text{CIP,heme,}2Ca^{2+}]_N \rightleftharpoons [\text{apoCIP,}2Ca^{2+}]_{N'} + \text{heme} \rightleftharpoons CIP_U + 2Ca^{2+} + \text{heme} \quad (9)$$

Heme-extraction from peroxidases can be carried out only at pH ≤ 2, presumably as a result of protonation of active site histidines. At pH 12.1 the more likely mechanism of protein unfolding involves tyrosine ionization and lysine and

arginine deprotonization. In urea the initial unfolding is a result of the binding of urea to the protein surface (Timasheff, 1993). At high temperature the entropy of the unfolded state is predominant. Reaction 8 is also in agreement with a late release of heme from the similar horseradish peroxidase HRP C as observed by CD and fluorescence spectroscopy in GdmCl equilibrium studies (Pappa & Cass, 1993). On the other hand heme can only bind specifically to the folded apoCIP. This is well-known from reconstitution of apoperoxidases with heme and from the folding of fully active horseradish peroxidase from recombinant polypeptide horseradish peroxidase expressed in *E. coli* (Smith et al., 1990; Gazarian et al., 1994). We conclude that the unfolding/refolding reactions of CIP, which we monitored as the change in Soret absorbance to indicate the state of heme, will be representative for protein unfolding/refolding reactions following the two-state model (eq 7). Fungal and plant peroxidases are particularly suited for such studies due to the built-in heme reporter group and the structural calcium ions that permit the trapping of the unfolded form and, hence, determination of the unfolding rate or time constants independently of refolding. The sensitivity of CIP to salt, in particular at high pH, might, however, be unique to fungal and plant peroxidases, as both classes are stabilized by two Ca^{2+}, each of which is bound to two carboxylate side chains. Furthermore, a buried salt bridge (Asp114 to Arg140 in CIP) is invariant in the plant peroxidase superfamily (Welinder et al., 1992; Welinder, 1992). In the work of Cherry et al. (1995) a destabilizing repulsion between Glu214 and Glu239 in CIP was abolished by changing Glu239 to a neutral or basic residue.

In conclusion, the present work shows that the mechanisms of CIP unfolding/refolding are similar in urea and at high temperature, as the transition state in both cases is more similar to the N than the U state. The reactions at high pH are different and suggest a transition state closer to the U state. The data also suggest that *in vitro* folding of recombinant fungal or plant peroxidases will be markedly enhanced by glycerol in the folding medium and that the ionic strength should be kept low.

ACKNOWLEDGMENT

The expert technical assistance of Y. B. Larsen and C. Jönsson is greatly appreciated.

REFERENCES

Ahern, T. J., & Klibanov, A. M. (1985) *Science 228*, 1280−1283.

Andersen, M. B., Hsuanyu, Y., Welinder, K. G., Schneider, P., & Dunford, H. B. (1991a) *Acta. Chem. Scand. 45*, 206−211.

Andersen, M. B., Hsuanyu, Y., Welinder, K. G., Schneider, P., & Dunford, H. B. (1991b) *Acta. Chem. Scand. 45*, 1080−1086.

Arnao, M. B., Casas, J. L., del Rio, J. A., Acosta, M., & Garcia-Canovas, F. (1990) *Anal. Biochem. 185*, 335−338.

Baunsgaard, L., Dalbøge, H., Houen, G., Rasmussen, E. M., & Welinder, K. G. (1993) *Eur. J. Biochem. 213*, 605−611.

Brandts, J. F. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 213−287, Marcel Dekker, Inc., New York.

Cherry, J., Lamsa, M., Vind, J., Schneider, P., & Pedersen, A. (1995) *Proceedings of the International Workshop on Peroxidase Biotechnology and Application* Book of Abstracts (Greppin, H., & Egorov, A., Eds.) p 8, Lomonosov Moscow State University, Russia, and University of Geneva, Switzerland.

Cleland, J. L., & Wang, D. I. C. (1990) *Bio/Technol 8*, 1274−1278.

Creighton, T. E. (1992) *Protein Folding*, W. H. Freeman and Company, New York.

Dalbøge, H., Jensen, E. B., & Welinder, K. G. (1992) Pat. Appl. WO 92/16634.

Finn, B. E., Chen, X., Jennings, P. A., Saalau-Bethell, S. M., & Matthews, C. R. (1993) in *Protein Engineering: A Practical Approach* (Rees, A. R., Sternberg, M. J. E., & Wetzel, R., Eds.) pp 167−189, IRL Press, Oxford.

Gazarian, I. G., Doseeva, V. V., Galkin, A. G., & Tishkov, V. I. (1994) *FEBS Lett. 354*, 248−250.

Fukuyama, K., Kunishima, N., Amada, F., Kubota, T., & Matsubara, H. (1995) *J. Biol. Chem. 270*, 21884−21892

Imoto, T., Yamada, H., & Ueda, T. (1987) *J. Mol. Biol. 190*, 647−649.

Kjalke, M., Andersen, M. B., Schneider, P., Christensen, B., Schülein, M., & Welinder, K. G. (1992) *Biochim. Biophys. Acta 1120*, 248−256.

Kunishima, N., Fukuyama, K., Matsubara, H., Hatanaka, H., Shibano, Y. Amachi, T. (1994) *J. Mol. Biol. 235*, 331−344.

Levitt, M. (1981) *J. Mol. Biol. 145*, 251−263.

Limongi, P., Kjalke, M., Vind, J., Tams, J. W., Johansson, T., & Welinder, K. G. (1995) *Eur. J. Biochem. 227*, 270−276.

Mann, C. J., Shao, X., & Matthews, R. (1995) *Biochemistry 34*, 14573−14580.

Pappa, H. S., & Cass, A. E. G. (1993) *Eur. J. Biochem. 212*, 227−235.

Pedersen, A. H., & Carlsen, S. (1994) in *The Thirteenth International Symposium on the Life Sciences*, October 25−28, 1994, Kyoto, Japan, Book of Abstracts, pp 24−27.

Petersen, J. F. W., Kadziola, A., & Larsen, S. (1994) *FEBS Lett. 339*, 291−296.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−241.

Schmid, F. X. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 197−242, W. H. Freeman and Company, New York.

Schmid, F. X. Mayr, L. M., Mucke, M., & Schönbrunner, E. R. (1993) *Adv. Protein Chem. 44*, 25−66.

Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C., Thorneley, R. T. F., & Burke, J. F. (1990) *J. Biol. Chem. 265*, 13335−13343.

Smulevich, G., Feis, A., Focardi, C., Tams, J., & Welinder, K. G. (1994) *Biochemistry 33*, 15425−15432.

Tams, J. W., & Welinder, K. G. (1995) *Anal. Biochem. 228*, 48−55.

Tanford, C. (1970) *Adv. Protein Chem. 24*, 2−93.

Teale, F. W. J. (1959) *Biochim. Biophys. Acta 35*, 543.

Timasheff, S. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.

Timasheff, S. N., & Arakawa, T. (1988) in *Protein Function* (Creighton, T. E., Ed.) pp 331−348, IRL Press, Oxford, New York, Tokyo.

Veitch, N. C., Tams, J. W., Vind, J., Dalbøge, H., & Welinder, K. G. (1994) *Eur. J. Biochem. 222*, 909−918.

Vind, J. (1994) Ph.D. Thesis, University of Copenhagen, Denmark.

Volkin, D. B., & Klibanov, A. M. (1989) in *Protein Function* (Creighton, T. E., Ed.) pp 1−22, IRL Press, Oxford, New York, Tokyo.

Welinder, K. G. (1992) *Curr. Opin. Struct. Biol. 2*, 388−393.

Welinder, K. G., & Andersen, M. B. (1993) *Pat. Appl.* WO 93/24618.

Welinder, K. G., & Tams, J. W. (1995) in *Carbohydrate Bioengineering*, Proceedings of an International Conference, Elsinore, Denmark, 23−26 April 1995 (Petersen, S. B., Svensson, B., & Pedersen, S., Eds.) pp 205−210, Elsevier, Amsterdam.

Welinder, K. G., Mauro, J. M., & Nørskov-Lauritsen, L. (1992) *Biochem. Soc. Trans. 20*, 337−340.

Wetlaufer, D. B., & Xie, Y. (1995) *Protein Sci. 4*, 1535−1543.

BI953067L