

Cis-trans isomerization is rate-determining in the reactivation of denatured human carbonic anhydrase II as evidenced by proline isomerase

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The refolding of human carbonic anhydrase II is a sequential process. The slowest step involved is the recovery of enzymic activity ($t_{1/2}$ =9 min). Kinetic data from 'double-jump' measurements indicate that proline isomerization might be rate determining in the reactivation of the denatured enzyme. Proof of this is provided by the effect of proline isomerase on the reactivation kinetics: the presence of isomerase during reactivation lowers the half-time of the reaction to 4 min, and inhibition of proline isomerase completely abolishes this kinetic effect. A similar acceleration of the refolding process by proline isomerase is also observed for bovine carbonic anhydrase II, in contrast to what has previously been reported. In human carbonic anhydrase II there are two *cis*-peptidyl-Pro bonds at Pro³⁰ and Pro²⁰². Two asparagine single mutants (P30N and P202N) and a glycine double mutant (P30G/P202G) were constructed to investigate the role of these prolines in the rate limitation of the reactivation process. Both in the presence and absence of PPIase the P202N mutant behaved exactly like the unmutated enzyme. Thus, *cis-trans* isomerization of the Pro²⁰² *cis*-peptidyl bond is not rate determining in the reactivation process. The mutations at position 30 led to such extensive destabilization of the protein that the refolding reaction could not be studied.

cis-trans Isomerization; Peptidyl proline isomerase; Carbonic anhydrase; Refolding; Reactivation; Site-directed mutagenesis

1. INTRODUCTION

Many small proteins exhibit slow and complex refolding kinetics, and in some cases this is assumed to be due to the presence of proline residues in the polypeptide chain. The rate-limiting step in this refolding is believed to be a proline imide bond isomerization [1–3]. In the random-coil state of proteins, proline is the only amino acid that is known to exist in a *cis-trans* isomeric equilibrium mixture, in which a *cis* form amounts to approximately 20%. Recently, one has observed proline isomerization in the native state of staphylococcal nuclease and calbindin [4–6]. However, in the native state each proline is usually present in only one isomeric form at each position with dominance of the *trans* form. Thus, an isomerization of the prolines in the non-native conformation normally has to occur prior to or during the refolding process of a denatured protein. The time for this isomerization reaction is of the order ten to

several hundred seconds, i.e. the same amount of time required as for slow protein-folding reactions [7].

Bovine carbonic anhydrase II (BCA II) exhibits complex refolding kinetics with fast and slow kinetic stages [8–12]. Using the 'double-jump' technique (i.e. the delay-time dependence in the denatured state of the refolding of a protein), evidence was recently put forward that the slow step in the refolding of the bovine enzyme was due to proline *cis-trans* isomerization [13].

Human carbonic anhydrase II (HCA II) has been shown to undergo a sequential refolding process, in which the last step is the recovery of enzymic activity with a half-time of 9 min [14]. This is a comparatively slow process, indicating that proline *cis-trans* isomerization might be rate-determining.

In order to obtain direct proof of a rate-limiting proline isomerization in the folding process of the enzyme, the effect of peptidyl-prolyl *cis-trans* isomerase (PPIase) on the folding kinetic was investigated.

In HCA II there are two *cis*-peptidyl-Pro bonds (Pro³⁰ and Pro²⁰²) in the native state [15]. The reisomerization of these *cis*-prolines from the *trans* isomer dominating in the denatured state, might be a major factor limiting the rate of refolding. Considering this, we decided to replace these prolines by site-directed mutagenesis.

Abbreviations: GuHCl, Guanidine-HCl; BCA II, bovine carbonic anhydrase II; HCA II, human carbonic anhydrase II; PPIase, peptidyl-prolyl *cis-trans* isomerase.

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2. MATERIALS AND METHODS

2.1. Chemicals

Guanidine-HCl (GuHCl; sequential grade purchased from Pierce) was made metal-free by extraction with dithizone (7 mg/l) in CCl_4 . The concentration of GuHCl was determined refractometrically [16]. Cyclosporin A (Sandimmun) was a gift from Sandoz Ltd.

2.2. Enzyme preparations

Human erythrocyte carbonic anhydrase II (HCA II) was initially purified by affinity chromatography, according to Khalifah et al. [17] and then further chromatographed on a DEAE-cellulose column, according to Henderson and Hendriksson [18]. Bovine erythrocyte carbonic anhydrase II (BCA II) was prepared as previously described [19]. For in vitro site-directed mutagenesis, we used the method described by Kunkel [20], based on host-cell deficiency in the enzymes dUTPase (*dut*⁻) and uracil *N*-glycosylase (*ung*⁻). For production of the enzyme variants we used the expression plasmid pCA [21], kindly provided by Carol Fierke, Duke University, Durham, USA. The enzyme mutants were purified by affinity chromatography [17].

An extract of peptidyl-propyl *cis-trans*-isomerase (PPIase) from pig kidney cortex was prepared as described by Fischer et al. [22]. The subsequent chromatographic purification was performed according to Takahashi et al. [23] with minor modifications: the chromatographic resins DEAE-Sephacel and Sephacryl S-200 were replaced by DEAE-cellulose DE 23 and Sephacryl S-100, respectively. The progress of purification of PPIase was analyzed by measurements of enzyme activity, using the peptide Succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (Boehringer) as a substrate in a coupled assay with α -chymotrypsin (Boehringer) [23]. SDS-electrophoresis analyses were performed in a Mini-PROTEAN II dual slab cell (Bio-Rad) using the electrophoresis calibration kit for molecular weight determination of low-molecular-weight proteins (Pharmacia) as a reference. The protein concentration was measured spectrophotometrically using $\epsilon_{280\text{ nm}} = 8730\text{ M}^{-1}\text{cm}^{-1}$, which was calculated according to Gill and von Hippel [24].

2.3. Stability measurements

The stabilities of the enzyme forms ($8.3\text{ }\mu\text{M}$, 0.25 mg/ml) were determined by incubation for 24 h at 23°C in various concentrations of GuHCl buffered with 0.1 M Tris- H_2SO_4 , pH 7.5. The transition was followed by assaying the CO_2 -hydration activity and the absorbance change, measured as the $A_{292\text{ nm}}/A_{260\text{ nm}}$ ratio, as recently described [25].

2.4. Kinetic measurements

The denaturation of HCA II ($14.0\text{ }\mu\text{M}$, 0.42 mg/ml) was studied in 5 M GuHCl, 0.1 M Tris- H_2SO_4 , pH 7.5 at 23°C . The extent of the denaturation reaction was measured as the change in CO_2 -hydration activity and the change in UV absorbance.

The absorbance change was monitored at 292 nm (Perkin Elmer Lambda 5; 1 cm cuvettes). The enzyme was added manually to the sample cell containing the denaturant. The blank contained the denaturant but no enzyme. Native enzyme in 0.1 M Tris- H_2SO_4 , pH 7.5, was used as a reference.

The reactivation kinetics were registered after denaturation of HCA II ($14.0\text{ }\mu\text{M}$) in 5 M GuHCl for various incubation times. Reactivation was achieved by rapidly diluting to 0.3 M GuHCl and a protein concentration of $0.83\text{ }\mu\text{M}$ (0.025 mg/ml) [26]. All solutions were buffered with 0.1 M Tris- H_2SO_4 , pH 7.5. The renaturation reaction was monitored by measuring the recovery of CO_2 -hydration activity. The same procedure was carried out for BCA II, although 0.5 M GuHCl was used during the reactivation.

Aliquots were withdrawn during the renaturation and denaturation and transferred to the CO_2 -hydration assay solution containing 2.0 ml of 25 mM veronal- H_2SO_4 , pH 8.2, 16 mM Bromothymol blue and 0.5 mM EDTA. The reaction was started by adding 2 ml of saturated CO_2 solution, and the final volume and temperature of the assay solution were 5 ml and 0°C . Under these conditions, no reactivation or further inactivation of the enzyme occurs in the assay medium. Thus, a control

sample which was 50% denatured showed no change in activity after incubation for up to 20 min in the assay solution prior to the addition of CO_2 . This made it possible to collect samples for assay from the early phase of the denaturation and reactivation processes. The CO_2 -hydration assay and the calculations were performed according to Rickli et al. [27].

To investigate the effects of PPIase on the reactivations kinetics, $9.6\text{ }\mu\text{M}$ PPIase was added at the onset of the reaction; this was accomplished by including PPIase in the dilution solution. When studying the effect on inhibiting the action of PPIase on the refolding kinetics, both PPIase and cyclosporin A ($25\text{ }\mu\text{M}$) were included in reactivation-dilution solution.

3. RESULTS AND DISCUSSION

Proline *cis-trans* isomerization reactions are generally slow compared to the amount of time required to completely denature proteins in 5 M GuHCl. Hence, the peptide bonds at prolines retain their native conformation for some time, even in the denatured state, before *cis-trans* equilibrium is reached. Therefore, if *cis-trans* isomerization is a rate-determining step, proteins will refold much faster when renaturation is initiated soon after the completion of denaturation.

To discover whether proline isomerization might limit the rate of the renaturation process of denatured HCA II, the reactivation kinetics were measured after various delay times in the denatured state (5 M GuHCl).

3.1. Double-jump kinetic studies of the reactivation of HCA II

The time required to reach complete denaturation, as judged by the change in absorbance at 292 nm or by inactivation of the enzyme, was determined. This was

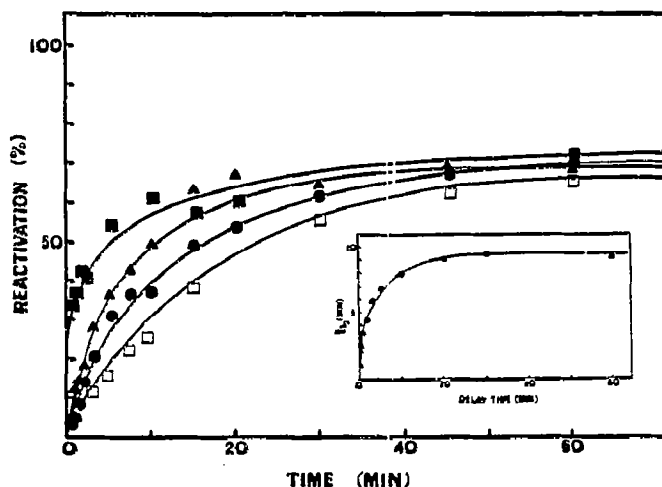


Fig. 1. Reactivation of HCA II after different delay times in the unfolded state. HCA II ($14.0\text{ }\mu\text{M}$, 0.42 mg/ml) was denatured for various times in 5.0 M GuHCl in 0.1 M Tris- H_2SO_4 , pH 7.5. Reactivation was initiated by rapid dilution to 0.3 M GuHCl and a protein concentration of $0.83\text{ }\mu\text{M}$ (0.025 mg/ml). Temp. 23°C . Delay-times in the denatured state: (■) 15 s; (▲) 2 min; (●) 10 min; (□) 1 h. For clarity only 4 experiments are shown in the main figure. The inset shows the half-times of reactivation obtained for various delay-times in the denatured state.

done after manual mixing, and the first point of measurement was obtained after 12 s of denaturation. Within this period of time the protein is fully denatured, according to the parameters tested. The observed change in $A_{292\text{ nm}}$ after 12 s of denaturation corresponds to a $\Delta\epsilon_{292\text{ nm}}$ of $-9150\text{ M}^{-1}\cdot\text{cm}^{-1}$, which is in agreement with the value obtained after 24 h of denaturation.

After this control experiment, the protein was allowed to denature for various lengths of time (15 s–2 h) in 5 M GuHCl, whereupon the kinetics of reactivation were studied (Fig. 1). The kinetics of reactivation are markedly affected by the delay-time of the protein in the denatured state. The half-time of reactivation is increased from 1 min to 9 min, when the time of denaturation is varied from 15 s to 1 h (inset in Fig. 1). These results indicate that *cis-trans* isomerization limits the rate of renaturation.

3.2. Effect of PPIase on the reactivation of HCA II

To confirm that the observed kinetic effects are due to *cis-trans* isomerization, the reactivation process was monitored in the presence and absence of PPIase. HCA II was denatured for 30 min in 5 M GuHCl to reach *cis-trans* equilibrium, and PPIase was added to the dilution buffer used to initiate refolding. In the presence of PPIase the half-time of reactivation of denatured HCA II was lowered to 4 min as compared to 9 min in the absence of PPIase (Fig. 2). Addition of cyclosporin A, which is a specific inhibitor of PPIase [23], completely abolishes the observed PPIase-mediated acceleration of the reactivation process (Fig. 2). These experiments definitely show that proline isomerization reactions limit the rate of HCA II refolding. However, PPIase cannot

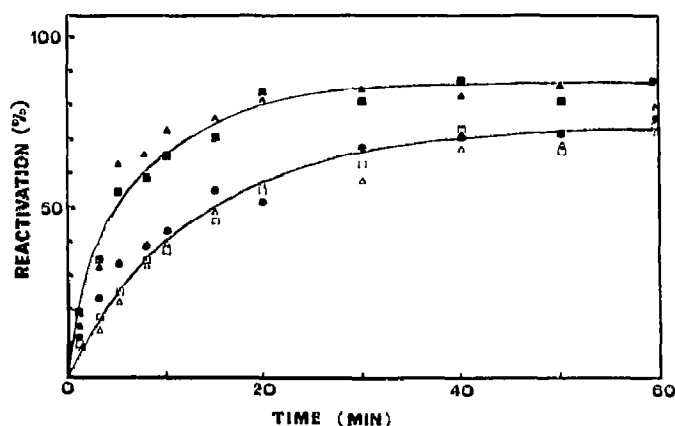


Fig. 2. Effect of PPIase on the reactivation of HCA II and a P202N mutant of HCA II. The native and mutant enzymes (0.42 mg/ml) were denatured for 30 min in 5.0 M GuHCl in 0.1 M Tris- H_2SO_4 , pH 7.5. Reactivation was initiated by rapid dilution of 0.3 M GuHCl and a protein concentration of 0.025 mg/ml (0.83 μM). PPIase and cyclosporin A were added to the reactivation dilution solution. Temp. 23°C. Reactivation kinetics for: (—□—) HCA II; (—■—) HCA II with PPIase (9.6 μM); (●) HCA II with PPIase (9.6 μM) and cyclosporin A (25 μM); (△) P202N mutant; (▲) P202N mutant with PPIase (9.6 μM).

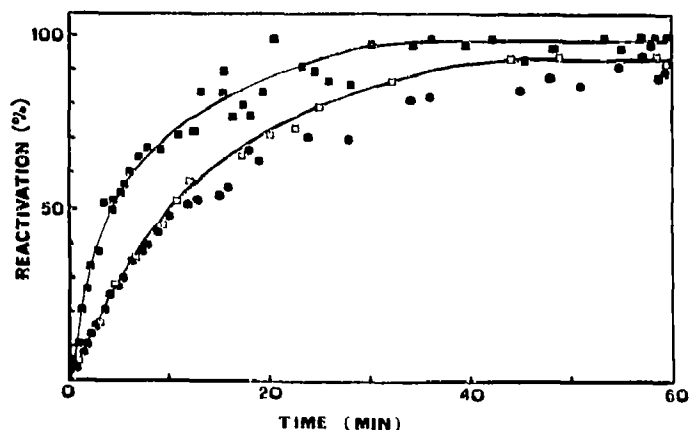


Fig. 3. Effect of PPIase on the reactivation of bovine CA II. The enzyme (0.25 mg/ml) was denatured for 30 min in 5.0 M GuHCl in 0.1 M Tris- H_2SO_4 , pH 7.5. Reactivation was initiated by rapid dilution to 0.5 M GuHCl and a protein concentration of 0.025 mg/ml (0.83 μM). PPIase and cyclosporin A were added to the reactivation dilution solution. Temp. 23°C. Reactivation kinetics for: (—□—) bovine CA II; (—■—) bovine CA II with PPIase (9.6 μM); (●) bovine CA II with PPIase (9.6 μM) and cyclosporin A (25 μM).

act efficiently on all the peptidyl-Pro bonds, since the half-time was only decreased to 4 min and not to 1 min or less, which might otherwise be expected. It seems probable that PPIase can only act on Pro residues that are exposed on the surface of the HCA II molecule during the folding process.

The yield of reactivated HCA II is also higher when active PPIase is present, indicating that formation of incorrect proline conformers during refolding at least partly explains why 100% reactivation is not normally achieved. In this context it is noteworthy that polyethylene glycol (PEG) has recently been shown to both enhance the rate of refolding of bovine carbonic anhydrase II and prevent aggregation [28]. In the study cited it was hypothesized that by interacting with PEG the enzyme would proceed through the slow steps of proline isomerization at a faster than normal rate, and an early folding intermediate would thereby avoid association leading to aggregation. This interpretation of the aggregation process occurring during refolding is supported by our observation that PPIase exerts a positive effect on the yield of active enzyme.

3.3. Effect of PPIase in the reactivation of bovine CA II

In contrast to our results with HCA II, Semisotnov et al. [13] were not able to show that PPIase had any effect on the refolding kinetics of bovine carbonic anhydrase II (BCA II). The negative results of these researchers prompted our investigation of the role of PPIase in the refolding of BCA II. We found that PPIase accelerated the reactivation kinetics of BCA II (Fig. 3) to the same degree as it did the reactivation of HCA II (Fig. 2). The problem of aggregation during refolding is less pronounced with the bovine enzyme

Table I
Midpoint concentrations of denaturation in GuHCl (M).

	CO ₂ activity ^a	A ₂₉₂ /A ₂₆₀ ^b
Cloned HCA II	0.93	0.94
Erythrocyte HCA II	0.91	0.94
P202N	0.45	0.45
P30N	<0.1	<0.1
P30G/P202G	<0.1	<0.1

^aThe denaturation transition was monitored by enzyme activity.

^bThe first of two observed transitions registered by absorbance measurements.

than with HCA II, leading to almost complete reactivation both in the absence and presence of PPIase.

Considering our results, and since Semisotnov et al. [13] did not provide the experimental details of their PPIase experiment, it is difficult to explain their negative results in regard to a PPIase-mediated catalysis of the refolding of BCA II.

3.4. Studies on Pro-mutants of HCA II

In the native state, HCA II contains two *cis*-peptidyl-Pro bonds (Pro³⁰ and Pro²⁰²) out of a total of 17 Pro residues [15]. These two *cis*-prolines can be suspected to contribute to the rate determination of the reactivation process. Furthermore, all three of the cytosolic isoenzymes, as demonstrated for HCA I [26], HCA II [26] and bovine CA III (unpublished data, manuscript in preparation), exhibit similar, slow reactivation kinetics, indicating a similar folding mechanism. Therefore, the most important Pro residues involved in rate limiting *cis-trans* isomerizations might be present among the 7 invariant Pro residues in these three isoenzymes (Pro in pos. 19, 21, 30, 181, 201, 202 and 250, [29]); notably Pro³⁰ and Pro²⁰² are among them.

Replacement of Pro³⁰ and Pro²⁰² by site-directed mutagenesis could therefore give rise to an observable effect on the refolding kinetics. A glycine double mutant (P30G/P202G) and two asparagine single mutants (P30N and P202N) were constructed for this purpose. The midpoint concentrations of denaturation caused by GuHCl for the mutants are given in Table I.

The enzyme was highly destabilized by the glycine double mutations as well as by the asparagine mutation in position 30 (Table I). We have previously shown that the stability of the native state of cloned HCA II is 7.9 kcal/mol in water [30]. Our result indicates that this stability must be almost completely lost due to these mutations. In a previous study, we observed that mutations in the neighbouring position 29 are critical for the stability of HCA II [25]. In the wild-type enzyme, Ser²⁹ participates in a hydrogen bond network, and the surrounding amino acids (Nos. 28–30) are evolutionarily invariant [29] and located in a rigid and compact substructure of the molecule [15]. In addition, these

amino acids are part of an open turn (Nos. 28–31, type VIb). The present study further demonstrates that substitutions of amino acids in this conservative region are very restricted. Replacement of the other *cis*-Pro (No. 202) gives rise to a more moderate destabilization (a lowering of the midpoint concentration of denaturation from 0.9 to 0.45 M GuHCl; Table I), despite localization in a reversed turn (Nos. 200–203, type VIa) [15]. Due to the low stability of the mutants P30G/P202G and P30N, it was impossible to use them in renaturation experiments. However, the P202N mutant was stable enough to allow such studies. The half-time of reactivation of the P202N mutant was 9 min (Fig. 2), which is identical to that of HCA II. Addition of PPIase gave rise to the same effect on refolding kinetics and recovery of CO₂-hydration activity for the P202N mutant as for the unmutated enzyme. Thus, the half-time of reactivation was decreased to approximately 4 min. *cis-trans* isomerization of the *cis*-peptidyl-Pro-202 bond does, apparently, not limit the rate of refolding as measured by the recovery of enzymic activity.

It can be assumed that *cis-trans* isomerizations of the Pro residues that are buried in the native state are the main contributing factors limiting the rate of refolding, because these residues are probably hidden very early in the folding process during formation of a compact state (molten globule) [31]. Rearrangements of amino acid residues in this state will, for steric reasons, probably be restricted and hindered. Pro³⁰ and Pro¹⁸¹ are the least accessible of the invariant Pro residues in the core of the native protein structure [15]. Unfortunately, substitutions at position 30 lead to such large destabilizations of the protein structure that kinetic studies of the refolding process are not feasible. Because of this, we cannot exclude the possibility that *cis-trans* isomerization at Pro³⁰ plays a role in the rate determination of the refolding process.

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