Proline Can Have Opposite Effects on Fast and Slow Protein Folding Phases

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ABSTRACT Proline isomerization is well known to cause additional slow phases during protein refolding. We address a new question: does the presence of prolines significantly affect the very fast kinetics that lead to the formation of folding intermediates? We examined both the very slow (10–100 min) and very fast (4 μs–2.5 ms) folding kinetics of the two-domain enzyme yeast phosphoglycerate kinase by temperature-jump relaxation. Phosphoglycerate kinase contains a conserved *cis*-proline in position 204, in addition to several *trans*-prolines. Native *cis*-prolines have the largest effect on folding kinetics because the unfolded state favors *trans* isomerization, so we compared the kinetics of a P204H mutant with the wild-type as a proof of principle. The presence of Pro-204 causes an additional slow phase upon refolding from the cold denatured state, as reported in the literature. Contrary to this, the fast folding events are sped up in the presence of the *cis*-proline, probably by restriction of the conformational space accessible to the molecule. The wild-type and Pro204His mutant would be excellent models for off-lattice simulations probing the effects of conformational restriction on short timescales.

INTRODUCTION

Complete refolding of many large globular proteins requires escape from misfolded states caused by proline isomerization. (Brandts et al., 1975). The high barrier for isomerization (16–20 kcal/mol in model compounds) results in characteristic times of 10–1000 s at room temperature (Brandts et al., 1975). In the unfolded protein, ~80% of the prolines are in the *trans* conformation, and 20% are in the *cis* conformation. Thus the presence of a *cis*-proline in the native structure results in a higher fraction of incorrectly isomerized molecules in the unfolded starting ensemble (Kiefhaber et al., 1990b; Texter et al., 1992). Mutagenesis studies have shown that specific proline residues can be assigned to slow recovery phases from misfolded states (Evans et al., 1987; Herning et al., 1991; Kelley and Richards, 1987; Kiefhaber et al., 1990a; Wood et al., 1988). The large literature on proline isomerization was reviewed recently by Balbach and Schmid (2000).

Although the role of prolines during recovery from misfolded states has been studied extensively, their effect on the fast initial collapse of proteins has not been studied. It is possible that some of the nonexponential kinetics that form fast-folding intermediates is caused by proline isomers: *trans* and *cis* ensembles that do not interconvert on a submillisecond timescale could collapse or form structure at different rates, and so prolines could manifest themselves on fast timescales also.

As a model system for the effect of proline residues on the early steps of protein folding, we study PGK. yPGK (Fig. 1) is a monomeric two-domain protein that reversibly catalyzes the transfer of the phosphate group between 3-phosphoglycerate and ATP (Watson et al., 1982). The amino acid residues important in this study are shown in Fig. 1. There are two tryptophans in the C-terminal domain (for fluorescent probing of the folding reaction). The highly conserved Pro-204 residue is the only *cis*-proline in yPGK (McHarg et al., 1999). It is located on the C-terminal side of the interdomain hinge. McHarg and co-workers have shown that the P204H mutant of yPGK retains ~85% of wild-type secondary structure content.

We consider the effect of eliminating the *cis*-proline via a P204H mutation. Refolding kinetics of the wild-type and mutant are initiated by a temperature jump, starting from the cold denatured state. We find that proline slows down recovery from misfolded states (minute timescale), but speeds up the formation of a compact intermediate (millisecond timescale). Thus *cis*-proline can have opposite effects on the accumulation of early intermediates and on the formation of large quantities of the native state late during folding. The nonexponential early-time kinetics are not altogether eliminated by the Pro204His mutation, indicating that there must also be other causes for the heterogeneity of the folding reaction at short times.

PGK refolding from the cold denatured state has been studied extensively, providing a benchmark for our work (Damaschun et al., 1993; Gast et al., 1993). Although cold denaturation is an apparent two-state process thermodynamically (Damaschun et al., 1998; Gast et al., 1995), refolding of yPGK was invariably found to proceed via an intermediate

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Abbreviations used: GuHCl, guanidine hydrochloride; DTT, 1,4-dithio-L-threitol; EDTA, ethylenediamine-tetraacetic acid disodium salt; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; IPTG, isopropyl β-D-1-thiogalactopyranoside; PGK, phosphoglycerate kinase; yPGK, yeast phosphoglycerate kinase; hisPGK, histidine tagged variant of yeast phosphoglycerate kinase.

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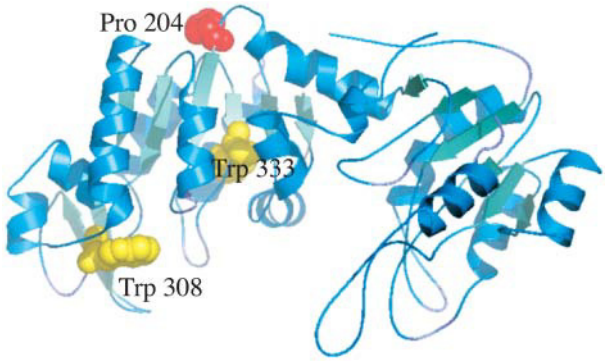

FIGURE 1 Three-dimensional structure of yPGK. Pro-204, Trp-308, and Trp-333 are shown space-filled (Watson et al., 1982).

(Beechem et al., 1995; Lillo et al., 1997; Ptitsyn et al., 1990). Fast temperature-jump measurements showed that this intermediate forms via nonexponential kinetics on a 0.01- to 10-ms timescale (Sabelko et al., 1999). Subsequently PGK takes minutes to refold to the native state (Betton et al., 1992; Gast et al., 1997; Otto et al., 1994). Very recently, the complications caused by two domains and by multiple tryptophan probes have been investigated in detail and were not found to cause any qualitative changes in the refolding kinetics (Osváth and Gruebele, submitted for publication).

MATERIALS AND METHODS

Enzymes and chemicals

All chemicals, antibiotics, and components needed for the bacterial culture media, potassium phosphate, IPTG, GuHCl, and AEBSF were obtained from Fisher Scientific (Chicago, IL), whereas DTT and EDTA were obtained from Sigma. Wild-type yPGK was obtained from Sigma and was used unmodified. It only served as a control for the wild-type His-tagged variant of the enzyme (hisPGK) expressed in *Escherichia coli* as described below. The far UV circular dichroism (CD) spectra (between 200 nm and 260 nm) of yPGK and hisPGK were identical within the 5% error of the measurement, indicating identical secondary structure content. Their stability and folding/unfolding cooperativity in GuHCl titrations were also found identical by far UV CD and integrated tryptophan fluorescence intensity measurement excited at 295 nm. Cold denaturation scans between −16°C and 10°C were found to be identical, monitored by CD at 222 nm, but fluorescence measurements excited at 295 nm showed some differences as detailed previously (Ervin et al., 2002).

Construction of the bacterial hisPGK expression system and of the P204H mutant

The yeast expression system of yPGK was originally developed by R. A. Hitzeman and granted to us by M. Mas (Hitzeman et al., 1980; Mas et al., 1987). The plasmid consists of the yPGK gene cloned in the YeP9T shuttle vector. The plasmid was amplified in the XL-1 Blue strain of *E. coli*, purified using QIAprep Miniprep kit (Qiagen, Valencia, CA), and digested with Hind III. The fragments were separated on agarose gel, and the middle (3.1 kbase) band of the three was excised and purified using QIAquick gel extraction kit. The purified fragment was ligated (T4 ligase from Promega, Madison, WI) into Hind III digested pET28 plasmid (Novagen, Darmstadt, Germany) and amplified in XL-1 Blue cells. Several colonies were tested, and plasmids containing the yPGK gene in the correct sense were selected based on the molecular weight of the fragments obtained after EcoR I (Invitrogen/Gibco, Carlsbad, CA) digestion. To the selected plasmid an Nde I cleavage site was added at the beginning of the PGK gene using Stratagene's QuickChange site-directed mutagenesis kit. After amplification, the mutant plasmid was digested with Nde I (Invitrogen/Gibco). The heavier fragment (the PGK containing pET28 plasmid) was separated from the removed, unnecessary 1.5 kbase DNA piece on agarose gel, purified, and religated. After amplification, this final plasmid was checked by sequencing and used for bacterial expression of His-tagged yPGK. The P204H mutation was added to the hisPGK gene using QuickChange site-directed mutagenesis kit. The presence of the mutation was checked by DNA sequencing.

Protein expression and purification

The wild-type and P204H mutant of hisPGK were expressed in the BL21-CodonPlus (DE3)-RP strain of *E. coli* obtained as supercompetent cell stock from Stratagene. Aliquots (5 ml) of overnight LB cultures started from single cell colonies were used to inoculate 1 L 2×YT media. All cultures contained 34 mg/L chloramphenicol and 30 mg/L kanamycin. After 4 h of growth in a shaker at 37°C, protein expression was induced by the addition of 1 mM IPTG, and then growth was continued under the same conditions for another 4 h. Cells were harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at −20°C until further use.

By inserting the PGK gene into the pET28 plasmid, the protein was automatically fused to a His-tag on its N-terminal. This made possible a His-affinity purification on Ni-NTA column (Qiagen). After the addition of roughly 0.3 mg AEBSF/60 g wet cell weight, cells were lysed by sonication and purified according to Qiagen's nondenaturing protocol without changes. hisPGK eluted between 25 and 40 mM imidazole. The fractions collected were checked for protein content and purity by denaturing polyacrilamide gel electrophoresis. The cleanest fractions were pooled and concentrated to 20–30 μM by ultrafiltration, extensively dialyzed at 4°C against 20 mM pH: 7.0 phosphate buffer, centrifuged for 20 min at 20,000 × g to remove any precipitate, flash frozen in liquid nitrogen, and stored at −20°C.

Solvent conditions

Unless otherwise noted, all samples were buffered in 50 mM, pH 6.2 potassium phosphate, 1 mM EDTA, 1 mM DTT aqueous solution for all experiments, and centrifuged for 20 min at 12,000 rpm using a SS-34 rotor (20,000 × g; Sorvall, Newtown, CT) before the measurements.

Steady-state and slow-folding/unfolding studies

Far UV CD spectra (200–250 nm), integrated fluorescence (330 nm cutoff filter, 295 nm excitation) and cold denaturation scans (−20°C to 20°C), and GuHCl titrations were recorded on a JASCO J-700 spectrometer equipped with a fluorescence accessory and automatic titrator. For the slow-folding experiments, CD data at 222 ± 1 nm were collected every 4 min.

Fast folding

Refolding processes faster than 2.5 ms were followed by time resolving changes in the tryptophan fluorescence decay profile (excited at 292 nm, detected with a 310–380 nm bandpass filter) (Ballew et al., 1996a,b) using a home-built laser temperature-jump apparatus equipped with a 500 ps rise-time photomultiplier detector (Ballew et al., 1996c; Gruebele et al., 1998). Jumps of 15°C in 10 nanoseconds were induced by a Raman shifted (1.54 μm) Nd:YAG laser pulse. The initial temperature was ~−12°C as monitored by a thermocouple, so refolding of PGK was initiated from the cold denatured state. The temperature-jump (T-jump) size was calibrated by measuring lifetime changes of pH 7 tryptophan solutions as a function of

both temperature and Raman pulse power. Ten folding kinetics runs of 2.5 ms were averaged. The 2.5-ms time window was achieved by measuring five 0.51-ms pieces of the kinetics with 0, 0.5, 1, 1.5, and 2 ms delay time after the T-jump under identical conditions and with the same sample, and combining them into one trace by using time overlaps in the data. The data are reported as χ_1 traces, as discussed in detail in Ervin et al. (2000):

$$f_{obs} = c_1(t)f_1 + c_2(t)f_2, \quad \chi_1(t) = c_1(t)/[c_1(t) + c_2(t)]. \quad (1)$$

The fluorescence signatures in the 20–25 μs and 480–500 μs ranges were arbitrarily chosen as the reference states 1 and 2 (where $\chi_1 = 0$ and 1, respectively), and $\chi_1(t)$ was determined by linear fitting of the data to Eq. 1. For a simple two-state mechanism, it has been shown that $\chi_1(t)$ is a single exponential decay, whereas heterogeneous kinetics produces nonexponential decays (Ervin et al., 2000). (Singular value decomposition should be used instead if the residuals to Eq. 1 become too large, but this was not necessary here (Ervin et al., 2000)).

RESULTS

Stability against GuHCl-induced unfolding

Fig. 2 *A* shows GuHCl titrations of wild-type and P204H mutant of hisPGK. Changes in secondary structure were monitored by CD at 222 nm. The P204H mutant clearly deviates from simple two-state behavior. Two steps are obvious in the mutant trace in Fig. 2 *A*. In the case of the wild-type, the two transitions were closer than 0.1 M GuHCl to each other, but the titration was asymmetrical. A fit to a two-state unfolding model yielded a significant residue

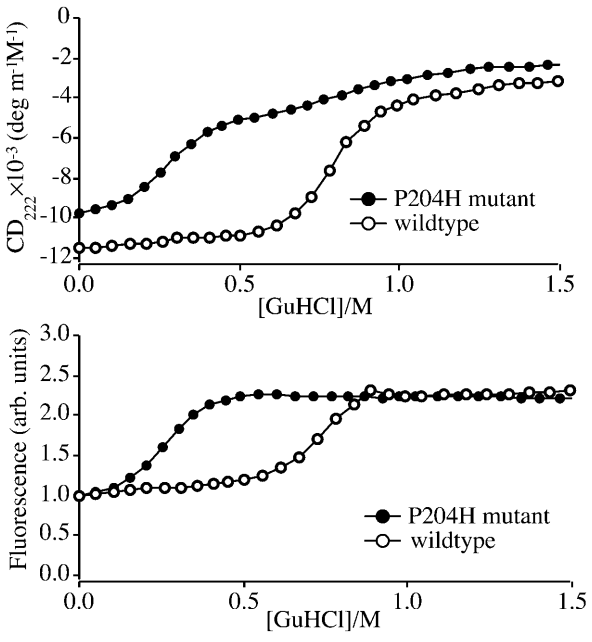

FIGURE 2 GuHCl titration of wild-type (*empty circles*) and P204H (*filled circles*) hisPGK monitored by mean residue ellipticity at 222 nm and by integrated fluorescence intensity excited at 295 nm. Emission intensities were normalized to the fluorescence of the folded proteins (0 M GuHCl, 20°C). Conditions: 50 mM potassium phosphate, 1 mM DTT, 1 mM EDTA, pH 6.2, 20°C. Protein concentration was 0.9 μM and 2.5 μM for the WT and Pro204His, respectively.

(data not shown). A model fit including a third state gave a residue within the noise level of the measurements for both mutant and wild-type. The stability data obtained for both enzymes are shown in the Table 1. The free energy differences between the native, intermediate, and unfolded states, as well as the population of these states as a function of GuHCl concentration, were extracted from the model fit (Fig. 3). The two tryptophans W308 and W333 are sensitive probes of conformational changes in the C-terminal domain. Comparison of Fig. 2, *A* and *B* shows that upon P204H mutation, the fluorescence trace follows the first step in the CD trace, linking that transition to the C-terminal domain.

Cold denaturation

Cold denaturation experiments were carried out with Pro204His hisPGK at 0 M, 0.1 M, 0.2 M of GuHCl and with wild-type hisPGK in 0.1 M, 0.2 M, 0.4 M GuHCl. The unfolding was monitored by CD at 222 nm (Fig. 4) and by tryptophan fluorescence intensity excited at 292 nm (Fig. 5). These measurements indicate that the stability of the Pro204His mutant against cold denaturation decreases roughly 7°C per 0.1 M GuHCl. In the case of the wild-type, the shift of the cold denaturation curves upon addition of the chaotropic salt is less pronounced, ~5.5°C per 0.1 M GuHCl. The dependence of the stability of the enzymes on temperature and denaturant concentration was used to find conditions where both the mutant and wild-type are destabilized to the same extent during the cold denaturation. This allows us to compare kinetics traces at the same final value of the folding equilibrium constant (same folding free energy).

At low GuHCl concentrations, cold denaturation of hisPGK can be fitted by a two-state model. Moreover, the CD spectra of neither protein show steps or asymmetry at temperatures where the fluorescence maximum of yPGK occurs. This does not rule out intermediates at higher free energy (at least 3–4 kT above the denatured or folded state, whichever is more populated). Such intermediates could show up in kinetics experiments, and in fact they do (see below).

Slow folding

Fig. 6 shows measurements of the slow phase of hisPGK unfolding and refolding for wild-type and the Pro204His mutant. Samples were equilibrated for 15 min before the temperature switch from 20°C (folded) to −15°C (cold denatured), allowed to evolve for 85 min, then refolded by a switch back from −15°C to 20°C. The slow unfolding kinetics can be fitted by a double exponential. The amplitude of the slow conformational change decreases considerably and the kinetics speed up in the mutant without *cis* proline. This implies that Pro-204 isomerization plays an important role in the formation of at least two

TABLE 1 Stability data extracted from the three-state model fits of the equilibrium GuHCl titration of wild-type and P204H mutant of hisPGK. The CD measurements used in this fit are shown in Fig. 2. The folded state is the reference state, so $\Delta G_1 = \Delta G_{I\text{-}F}$ and $\Delta G_2 = \Delta G_{U\text{-}F}$

	m_1 (kJ mol^{-1} M^{-1})	C_{m1} (M)	ΔG_1 (kJ mol^{-1})	m_2 (kJ mol^{-1} M^{-1})	C_{m2} (M)	ΔG_2 (kJ mol^{-1})
Wild-type	40.1 ± 1.3	0.783 ± 0.006	31.4 ± 1.2	52.1 ± 3.9	0.857 ± 0.04	44.6 ± 5.5
P204H	33.0 ± 2.5	0.277 ± 0.007	9.1 ± 0.9	51.1 ± 3.0	0.462 ± 0.16	23.6 ± 9.5

different ensembles of states during unfolding, in agreement with previous results in the literature (Evans et al., 1987; Maki et al., 1999; Walkenhorst et al., 1999). Upon refolding, mutant kinetics are essentially completed within the dead time of the temperature ramp, whereas the wild-type still exhibits completion of the kinetics. Thus the presence of the proline causes additional phases during unfolding, and slows down refolding kinetics, presumably because part of the native population can be formed only after escape from a *trans*-misfolded state.

Fast folding

Folding events faster than 2.5 ms were monitored by real-time tryptophan fluorescence lifetime changes after a laser-induced T-jump of preequilibrated samples from −12°C to +3°C. There appeared to be a slight effect of the preequilibration time on the exact shape of the relaxation kinetics, but it was not large enough to be quantified as a function of the delay time between achieving the initial temperature, and applying the T-jump. Relaxation to intermediate state(s) of the protein from the cold denatured state was found to be nearly complete in the wild-type after 2.5 ms, but still ongoing in the hisPGK mutant (Fig. 7). Thus removal of the proline residue slowed down the early-time folding kinetics of PGK.

Table 2 shows fits of the data to double exponential $(A_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2})$ and stretched exponential models $(A_0 + A_1 e^{-(t/\tau)^{\beta}})$. Neither the mutant nor the wild-type kinetics could be fitted satisfactorily to a single exponential decay. This was true over the full range from 4–2500 μs, but also over a truncated range from 20–2500 μs. (The latter was tested because some intrinsic relaxation events as slow as 15 μs under unfolded conditions have been observed previously (Sabelko, 2000).) Thus *trans-cis* isomerization of Pro-204 in hisPGK alone is not solely responsible for kinetic heterogeneity of early folding stages of the wild-type protein, which was also observed under slightly different conditions by Sabelko et al. (Sabelko et al., 1999). In that work, the formation of an intermediate ensemble of wild-type yPGK at 19°C was fitted to a stretched exponential with a lifetime of 3770 μs and $\beta \leq 0.83$ (or double exponentials with lifetimes of 176 and 4250 μs), which is to be compared with the hisPGK data in Table 2. It is worth noting that the double exponentials in Fig. 7 fit the experimental data slightly better than a stretched exponential. If the stretching is due to traps en route to the intermediate, this implies that there may be a small number of dominant traps. (A single trap would give

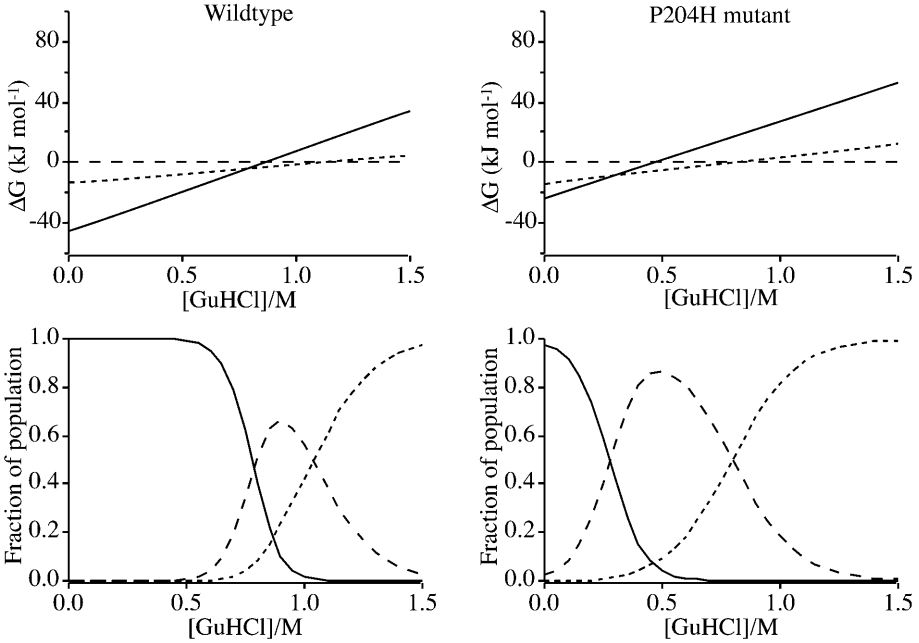

FIGURE 3 Variations in the free energies and populations of the folded, intermediate, and unfolded states of the wild-type and Pro204His. These data were obtained from a three-state model fit to the GuHCl equilibrium titrations shown in Fig. 2.

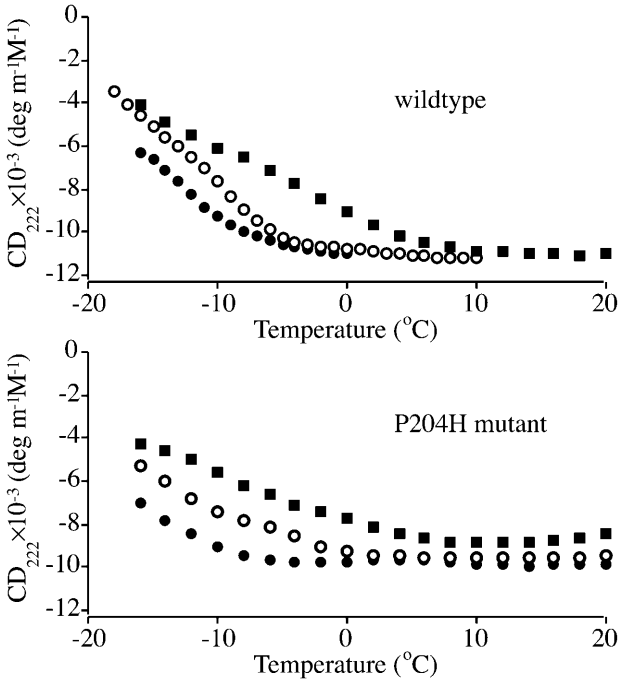

FIGURE 4 Cold denaturation of hisPGK wild-type in 0.1 M (*filled circles*), 0.2 M (*empty circles*), 0.4 M (*squares*) GuHCl and of P204H hisPGK in the presence of 0 M (*filled circles*), 0.1 M (*empty circles*), 0.2 M (*squares*) GuHCl. Changes in the secondary structure were followed by the mean residue elipticity at 222 nm. Samples contained 5 to 12 μM protein.

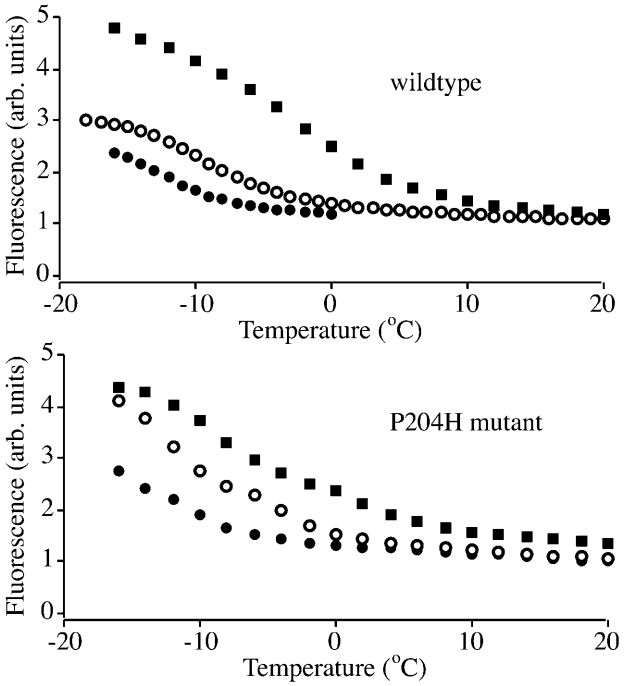

FIGURE 5 Cold denaturation of hisPGK wild-type in 0.1 M (*filled circles*), 0.2 M (*empty circles*), 0.4 M (*squares*) GuHCl and of Pro204His hisPGK in the presence of 0 M (*filled circles*), 0.1 M (*empty circles*), 0.2 M (*squares*) GuHCl. Conformational changes were followed by integrated fluorescence intensity excited at 292 nm. Fluorescence curves were normalized to the emission intensity of the folded protein species (0 M GuHCl, 20°C). Protein concentrations were between 5 and 12 μM.

a perfect biexponential fit with uncorrelated lifetimes for the two components; the fact that the biexponential lifetimes happen to closely mimic a stretched exponential indicates that there is probably more than one trap (Metzler et al., 1999).)

DISCUSSION

The formation of compact, partially structured, but still solvated folding intermediates requires less accurate registration of side-chain contacts than the native fold. The folding intermediate formed in the first few ms of PGK folding is such a case. Work by the Beechem and Mas groups has shown that yPGK collapses rapidly to a compact state, whereas work by Sabelko et al. narrows the timescale to 0.02–4 ms of nonexponential collapse dynamics. Hydrogen exchange experiments on a related bacterial PGK (Parker, 1996) have shown that native-like secondary structure is formed in many parts of the protein within milliseconds. Experiments by Ervin et al. using a ns resolution multichannel array detector have shown that the fluorescence spectrum of yPGK shifts by only ~3 nm in the intermediate (compared to an unfolded-native blue shift of ~30 nm), indicating that Trp side chains are not desolvated in the intermediate folding ensemble. Thus, such intermediates are expected to accommodate incorrect backbone dihedral angle distributions better than native states.

This idea is confirmed by our thermodynamic data (Table 1). The wild-type native state is stabilized by ~45 kJ/mole compared to the unfolded state, the P204H mutant only by 24 kJ/mole. In contrast, the intermediate stabilization energies are the same within measurement error ~14 kJ/mole). The thermodynamic penalty of the P204H mutation is thus introduced en route from the intermediate to the folded state. This observation can be rationalized by the following picture: His can largely sample the dihedral angles accessible to Pro in a loop, but the reverse is not true. This excess entropy of His manifests itself only upon native packing, not in the intermediate state.

Prolines generally cause additional slow phases during unfolding and refolding. The most dramatic effects are caused by *trans*-to-*cis* isomerization during the folding of proteins with native *cis* residues. Fig. 6 shows precisely such an effect in the expected direction. Slow kinetic measurements show that both the kinetics of the denaturation upon cooling, and of the refolding from the cold denatured state, proceed faster and in a smaller subpopulation in the Pro204His mutant than in wild-type hisPGK, although they are not completely eliminated. It has been proposed that the slow phase of the yPGK refolding from the GuHCl denatured state is independent of the proline isomerization (Semisotnov et al., 1991). If so, our results indicate that refolding of PGK

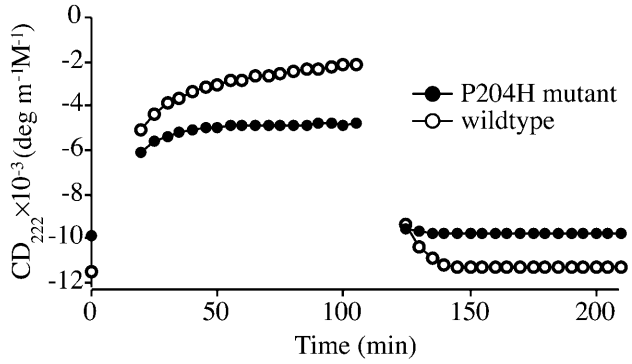

FIGURE 6 The slow phase of the cold denaturation and refolding of hisPGK WT in 0.4 M GuHCl (*empty circles*) and P204H in 0.2 M GuHCl (*filled circles*) was monitored by hand-mixing and measuring the mean residue elipticity at 222 nm. The sample temperature was dropped from 20°C to −15°C in 15 min. Slow conformational changes were followed for 90 min, then samples were heated to 20°C again and the slow refolding was traced for 90 min. Protein concentrations were 9.0 μM and 15.9 μM for the wild-type and P204H, respectively.

proceeds on significantly different parts of the free energy surface from the cold and GuHCl denatured states.

The relative size and rate of the slow phases can be rationalized by taking into account all the 17 Pro residues present in the protein. The most important of these is the Pro-204, the only *cis*-proline in the folded structure. Pro-204 will isomerize to *trans* with high probability in the denatured state, giving rise to about two-thirds of the slow unfolding phase, and to practically all of the resolvable slow refolding

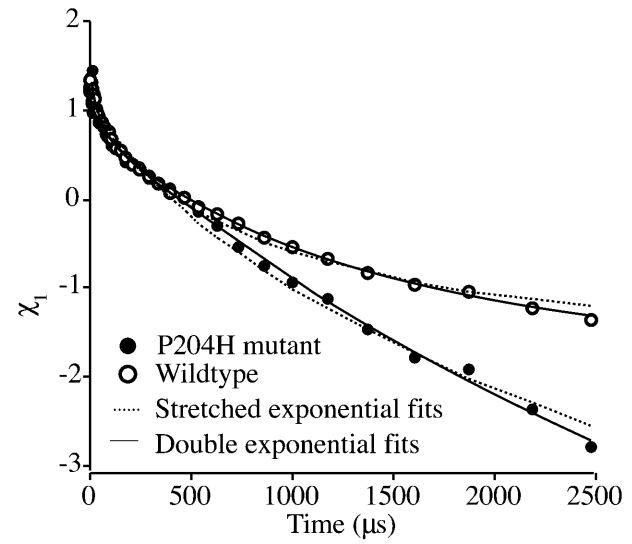

FIGURE 7 Fast refolding kinetics of wild-type (*empty circles*) and Pro204His (*filled circles*) hisPGK after a temperature jump from −12°C to +3°C. To induce a similar change in the folded and unfolded population during the cold denaturation and T-jump, the wild-type and Pro204His were destabilized by 0.15 M and 0.1 M GuHCl, respectively. For both enzymes solution concentrations ranged from 40 μM to 60 μM.

TABLE 2 Double exponential kinetic parameters for wild-type and Pro204His hisPGK kinetics fitted in the 4–2500 μs range. For comparison with Sabelko et al. (1999), a stretched exponential fit is also shown here and in Fig. 7

Biexponential	A_1	τ_1 (μs)	A_2	τ_2 (μs)
Wild-type	0.89 ± 0.04	90 ± 7	3.54 ± 0.02	1530 ± 80
P204H	0.58 ± 0.12	61 ± 12	7.80 ± 0.35	4030 ± 520
Stretched exp.	**Amplitude**	**τ (μs)**		**Streching factor β**
Wild-type	4.44 ± 0.53	2860 ± 80		0.57 ± 0.04
P204H	8.38 ± 0.16	4800 ± 450		0.71 ± 0.06

kinetics in the wild-type enzyme. The above complication is absent in the P204H mutant, so slower phases are reduced or absent. To explain the small remaining slow phase in the P204H mutant, we have to take into account the partial isomerization of the other 16 *trans* prolines into nonnative *cis* conformations during unfolding. Since the *trans* isomer is the more stable one, isomerization of these *trans*-prolines is less likely. All of them together account for roughly one-third of the slow unfolding kinetics and cause practically no hindrance on the several minutes timescale of the refolding process.

The opposite situation prevails on the <10 ms timescale, where presence of the proline speeds up equilibration of a folding intermediate. T-jump measurements show that refolding to an intermediate from the cold denatured state is still ongoing in the Pro204His mutant at 2.5 ms, but nearly complete in the Pro-containing wild-type. This can be explained by one of two related scenarios. In both scenarios, proline isomerization is at least three orders of magnitude slower than collapse. As a result, the refolding ensemble is partitioned into two preequilibrated conformational subensembles, one containing *cis* Pro-204, the other *trans* Pro-204.

In the first scenario, conformational restrictions speed up collapse irrespective of the proline isomerization state, by increasing backbone rigidity in the critical hinge between the N- and C-terminal domains. The His mutant simply introduces additional flexibility that increases the search time for a collapsed state. In the second scenario, the partitioned *cis* and *trans* configurational ensembles play different roles. The correctly isomerized *cis* subensemble collapses rapidly to a compact structure with native-like topology. The more flexible Pro204His mutant and the *trans* subensemble remains extended for a longer period. Because only the *cis* ensemble is ''active'' below 2 ms, the wild-type intermediate ensemble is formed more rapidly, but with a smaller population than in the Pro204His mutant. This effect could be responsible for the smaller amplitude of the wild-type kinetics in Fig. 7, although differences in Trp quenching could also be responsible.

The true scenario probably lies between these extremes, with the partitioning leading neither to completely equivalent collapse of the *cis*/*trans* subensembles, nor to collapse of just

one of the subensembles. It would be very interesting to simulate these differences in the early folding dynamics via an off-lattice model to shed more light on how different collapse dynamics in the *cis* and *trans* subensembles really are. In our experiments, we found a slight effect of the pre-T-jump equilibration duration on the early time dynamics, so *cis* and *trans* subensembles may indeed collapse differently.

Simulations also could address the important question of the structural properties of the intermediate ensemble. Many experiments have been conducted on whole yPGK and its N- and C-terminal fragments. The general conclusion is that upon GuHCl titration, the C-terminal domain unfolds first. Pro-204 is located in the hinge near the C-terminal of the protein, so one would expect its mutation to disrupt the C-terminus more. This is in agreement with our thermodynamic data in Figs. 2–5. Upon mutation, one of the GuHCl titration midpoints moves from ~0.8 M to ~0.25 M; similarly, the small fluorescence transition shifts to lower C_m. Furthermore, almost the entire fluorescence amplitude shifts to low C_m, whereas only about two-thirds of the CD amplitude does. Because the two fluorescent probes are both in the C domain (Trp-333 and Trp-308), these observations are entirely consistent with the Pro204His mutation destabilizing the C domain more than the N-domain, leading to approximately sequential unfolding of the domains during GuHCl denaturation.

Literature data at 0.7 M GuHCl indicate the presence of an intermediate during cold denaturation (Gast et al., 1993). Our cold denaturation titrations at ≤0.2 M GuHCl concentration indicate a loss of stability upon mutating Pro-204 to His but show no evidence of sequential unfolding behavior. Based on this observation, we posit that interdomain interactions are weakened in the presence of GuHCl, leading to more independent domain unfolding (sequential in the mutant), whereas cold denaturation retains a more concerted process with interaction among the domain hydrophobic surfaces and a larger stability gap between the folded state and other local minima in the free energy surface.

The data in Fig. 7 also set a lower time limit on interdomain interactions. Assuming that the differences between the wild-type and P204H kinetics are caused by altered domain interactions, they do not become significant until ~500 μs have passed. It is of course possible that the difference is entirely due to changes in C-domain folding, in which case domain interactions might not occur until much later than 500 μs (hence the lower limit).

In summary, the presence of a *cis*-proline residue leads not only to additional very slow phases but also modifies the ms and sub-ms dynamics in the two-domain protein PGK by speeding up the formation of an intermediate from at least one of the isomeric ensembles. However, removal of the *cis*-proline does not bring the fast folding kinetics closer to single exponential formation of the intermediate, so heterogeneous dynamics during collapse must have other causes, such as discussed in Sabelko et al. (1999).

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