Peptidyl-prolyl *cis-trans* isomerase does not affect the Pro-43 *cis-trans* isomerization rate in folded calbindin D_{9k}

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The calcium-binding protein calbindin D_{9k} has previously been shown to exist in two folded forms only differing in the proline *cis-trans* isomerism of the Gly-42–Pro-43 amide bond. This bond is located in a flexible loop connecting the two EF-hand Ca^{2+} sites. Calbindin D_{9k} therefore constitutes a unique test case for investigating if the recently discovered enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase) can affect the *cis-trans* exchange rate in a *folded* protein. The ^1H NMR saturation transfer technique has been used to measure the rate of interconversion between the *cis* and *trans* forms of calbindin in the presence of PPIase (PPIase:calbindin concentration ratio 1:10) at 35°C. No rate enhancement could be detected.

Proline *cis-trans* isomerism; Peptidyl-prolyl *cis-trans* isomerase; Cyclophilin; Calbindin; ^1H NMR; Saturation transfer

1. INTRODUCTION

Calbindin D_{9k} is a small (M_w 8500), globular, calcium-binding protein. A 0.23 nm resolution crystal structure [1] as well as a global fold based on two-dimensional ^1H NMR data [2] is available for the calcium saturated form of the bovine protein. According to these, calbinding comprises two so called EF-hands – i.e. helix-(Ca^{2+}-binding loop)-helix motifs [3] which in turn are connected by a flexible and solvent accessible linker loop, see Fig. 1. ^1H NMR data in addition show that the protein exists in two folded forms in the ratio 3:1. These forms are in chemical equilibrium and it has been shown conclusively that they only differ in the isomerization at Pro-43 [4] – a residue that resides in the solvent accessible linker loop. The interconversion between the *cis*-Pro-43 (minor) and *trans*-Pro-43 (major) forms of calbinding D_{9k} have been studied at elevated temperatures, which has provided an estimate of the rate of exchange at room temperature to be 0.2 s^{-1} [5]. This rate agrees well with values found for simple peptides [6].

The enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase) [7], shown to be identical to the cyclosporin A binding protein cyclophilin [8,9], can affect the *cis-trans* exchange rate of proline peptide bonds in oligopeptides [7,10] and (partially) unfolded proteins [11–15]. PPIase is not the only protein capable of influencing such an exchange. Recently a new protein, FK506-binding protein, have been purified and shown to have PPIase activity [16,17] although its rate enhancement for oligopeptides is 10–20-fold smaller than PPIase's. This protein also binds to a cyclic molecule with immunosuppressive properties, FK506.

Calbinding is rare in existing in two *folded* forms caused by proline *cis-trans* isomerism. The only other protein, so far discovered, showing a conformational heterogeneity known to be caused by proline *cis-trans* isomerism is staphylococcal nuclease [18–20]. Calbindin therefore constitutes a unique test case for investigating if PPIase also can enhance the *cis-trans* exchange rate in a *folded* protein. The *cis-trans* isomerization rate in folded calbinding was measured using the ^1H NMR saturation transfer technique [21]. In order to make the rate measurements more accurate these were performed on a calbindin preparation in which all amino acid residues but threonines are deuteriated. Thus, well separated resonances of corresponding *cis*-Pro43 and *trans*-Pro43 calbindin protons can be resolved from overlapping signals.

2. MATERIALS AND METHODS

2.1. *PPIase preparation*

To optimize the preparation of PPIase we have added our own ideas to the purification schemes previously published [7,9]; this will be reported in some detail.

Homogenization, pH-treatment and $(NH_4)_2SO_4$ precipitation were performed as described by Fischer et al. [7] using 500 g pig kidney as starting material. The $(NH_4)_2SO_4$ precipitated proteins were dialyzed against 50 mM Tris-HCl buffer, pH 8.5, and pumped onto a 5.2 × 18 cm DEAE-cellulose column using the same buffer. This was then eluated with a 1500 ml Tris-HCl pH-gradient starting with pH 8.5 and ending with pH 7.5 followed by 500 ml 1 M NaCl, Fig. 2A. The PPIase activity was measured using succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Calbiochem) under the conditions described by

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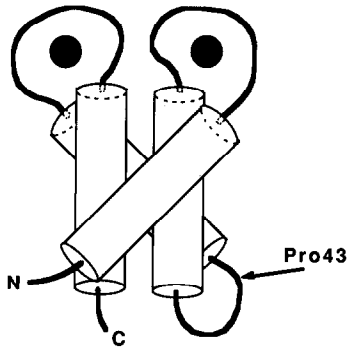

Fig. 1. Schematic representation of calbindin D_{9k}. α-Helical segments are represented by cylinders, loop regions by ribbons and Ca^{2+}-ions by spheres.

Takahashi et al. [9]. Fractions showing PPIase activity were pooled and concentrated to 20 ml upon which they were applied on a 3 × 190 cm Sephadex G-50 superfine column in 50 mM ammonium acetate, pH 6.0, Fig. 2B. Fractions of interest were again pooled, the pH adjusted to 7.5 and NaCl added to a total concentration of 1 M before pumping the sample onto a 1.5 × 15 cm phenyl-Sepharose column in 0.1 M Tris-HCl, 1 M NaCl, pH 7.5. The following eluation was carried out using a 400 ml concentration gradient starting with 0.1 M Tris-HCl, 1 M NaCl, pH 7.5 and ending with 0.1 M Tris-HCl, 0.4 M NaCl, pH 7.5 followed by 100 ml H_2O and then 100 ml 8 M urea, Fig. 2C. Fractions with PPIase activity were pooled and concentrated to 10 ml and applied onto the 3 × 190 cm Sephadex G-50 superfine column in 50 mM ammonium acetate, pH 6.0, Fig. 2D. PPIase fractions were finally pooled, dialyzed against H_2O and lyophilized. Protein and activity recovery during the purification is presented for each purification step in Table I and Fig. 3. It should be noted that the activity of our PPIase preparation is at least as high as the best preparations used in previous rate studies [11–15]. For the NMR experiment, 0.5 mg PPIase was dissolved in 0.5 ml D_2O, lyophilized and redissolved in 50 μl ultra puriss (99.5%) D_2O.

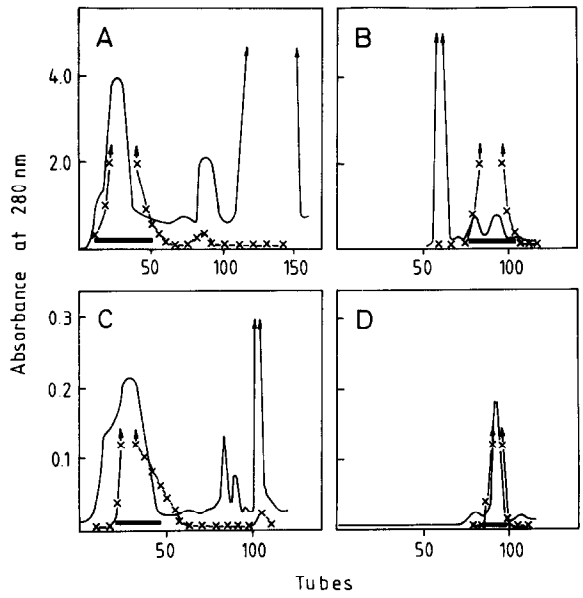

Fig. 2. Elution profiles from (A) DEAE-cellulose column, (B) Sephadex G-50, (C) phenyl Sepharose and (D) Sephadex G-50. (×—×) PPIase activity; pooled fractions are indicated with horizontal bars.

Table I

Purification of pig kidney PPIase

Purification step	Total protein (mg)	Recovered activity (%)
$(NH_4)_2SO_4$ precipitation	10600	100
DEAE-cellulose chromatography	950	63
Sephadex G-50 filtration	117	40
Phenyl-Sepharose chromatography	20	30
Sephadex G-50 filtration	3.8	15

2.2. *Calbinding preparation*

A special preparation of the protein calbindin in which all amino acid residues but threonines are deuteriated ([^{1}H]Thr, [^{2}H]calbindin) was expressed and purified according to Brodin et al. [22]. 2 mg protein was dissolved in 0.5 ml D_2O and the pH (not corrected for isotopic effects) was adjusted to 7.5 using μl amounts of NaOD and DCl. The sample was then lyophilized and redissolved in 520 μl ultra puriss D_2O.

2.3. *NMR measurements*

All NMR measurements were performed using a GE Ω 500 spectrometer and the acquired data were processed with the GE Ω software on SUN workstations. For each experiment 512 scans of 8192 complex data points were acquired using a spectral width of 3000 Hz. In order to improve the signal to noise ratio, the FID's were multiplied with an exponential function introducing a linebroadening of 2 Hz. Saturation transfer experiments [21] were performed by saturating the Thr-45 signal in the *cis*-form of [^{1}H]Thr, [^{2}H]calbindin with the decoupler at low power during 30 s. The decoupler power was sufficient to saturate the *cis* Thr-45 signal in one second. The longitudinal relaxation time T_1 was measured with the inversion recovery method [23] using a pulse repetition rate of 3 s plus the variable time delay (0.01–5 s). Since the residence time in each conformer is long compared to T_1, the exchange will have a negligible influence on T_1.

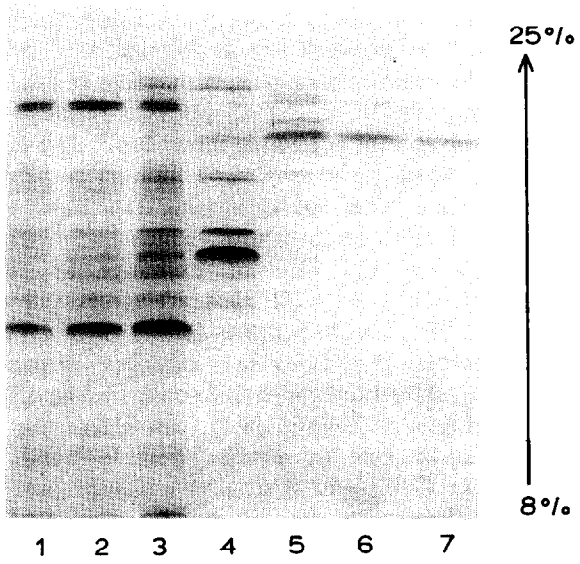

Fig. 3. Pharmacia prefabricated SDS-polyacrylamide 8–25% gradient gel, obtained on a Pharmacia Phast System, showing the different steps in the purification procedure. (1) After homogenization. (2) After pH-treatment. (3) $(NH_4)_2SO_4$ fraction. (4) PPIase fraction after DEAE-cellulose column. (5) PPIase fraction after Sephadex G-50. (6) PPIase-fraction after phenyl-Sepharose. (7) PPIase-fraction after Sephadex G-50.

3. RESULTS

The rate of *cis-trans* isomerization of Pro-43 in calbindin have previously been studied at pH 6.0 through high temperature lineshape studies [5]. This approach is, however, not applicable for rate studies in the presence of PPIase since, by contrast to calbindin, the heat stability of the enzyme is low (see Table II). Therefore, NMR experiments allowing exchange rate measurement at temperatures as low as 35°C, being the highest temperature PPIase can be exposed to for 4 h (the time of the experiment) and still retain most of its activity, had to be utilized. Furthermore, the pH optimum of PPIase activity is reported to be around 7.8 [7]. After confirming that the exchange rate, as determined by high temperature lineshape analysis, was not affected by a pH change from 6.0 to 7.5 the experiments were carried out at pH 7.5. The largest chemical shift difference for any methyl group between *cis*-Pro-43 and *trans*-Pro-43 calbindin is found for Thr-45 and amounts to 49 Hz at 11.74 T. In the isotopically labelled [^{1}H]Thr, [^{2}H]calbindin, these two signals are free from overlap and saturation transfer experiments could be performed as follows. The upfield shifted *cis*-Thr-45 signal was irradiated with the decoupler and the height of the *trans*-Thr-45 signal (I_{irr}) was compared to its height (I_{ref}) in a reference spectrum where the decoupler was placed 49 Hz downfield of the *trans* signal. If *trans*⟶*cis* exchange takes place during the irradiation this will reduce the height of the *trans* signal and knowing the longitudinal relaxation time, T_1, for the *trans* signal, the exchange rate can be calculated from

$$K_{trans \to cis} = \frac{1}{T_1^{trans}} \left(\frac{I_{ref}}{I_{irr}} - 1 \right)$$

At 40°C the peak height ratio (I_{irr}/I_{ref}) is 0.94 and using the T_1 value for the *trans* signal determined at 35°C to 0.92 s^{-1} the *trans*⟶*cis* exchange rate is calculated to 0.1 s^{-1}. This is to be compared with the value 0.2 s^{-1} *extrapolated* from the coalescence of the *cis* and *trans* Val-61 methyl signals above 70°C. After establishing that the experiment worked at 40°C the temperature was lowered to 35°C where the exchange rate in calbindin is just about too slow to affect the peak height. PPIase (0.5 mg) was then added to the calbindin solution, making the enzyme concentration 0.05 mM (10 mol%). Under our experimental conditions we should have been able to detect a twofold increase in the exchange rate. However, *no* effect on the exchange rate could be observed, as confirmed in Fig. 4.

Table II

Time dependence of PPIase activity (%)[a] at different temperatures

Time (min)	20°C	35°C	40°C	45°C
0	100	100	100	100
15	100	100	100	50
30	100	100	100	33
60	100	100	83	25
120	100	100	63	17
300	100	20	4	–
1440	80	–	–	–

[a] 100% = 1 μg PPIase converts 55 μg (total peptide) succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (1:1500 molar ratio) from the *cis* to *trans* form in 25 s at 20°C

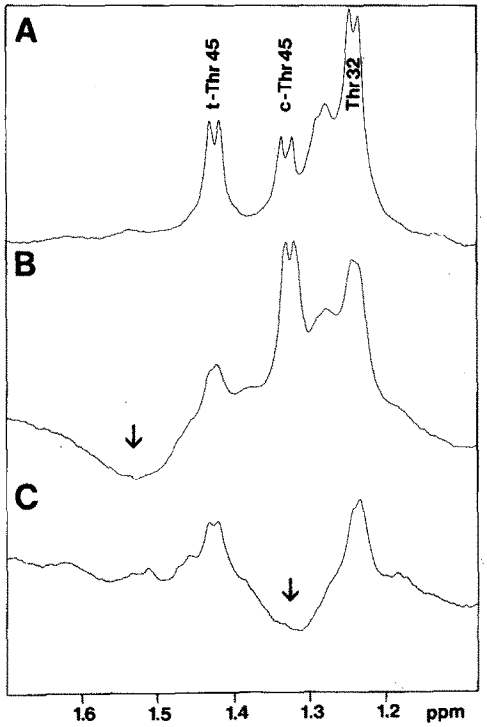

Fig. 4. ^{1}H NMR saturation transfer experiment. (A) A normal 1D ^{1}H NMR spectrum of [^{1}H]Thr, [^{2}H]calbindin. Additional proton intensity between 1.2 and 1.4 ppm, due to scrambling of the labelled threonine, make the *cis* signal appear higher than expected from the *cis/trans*, 1:3, population ratio. ^{1}H NMR saturation transfer experiment performed on a mixture of calbindin and PPIase (10:1 molar ratio) (B) with irradiation at 664 Hz (*cis* Thr-45 signal). (C) Reference spectrum with irradiation at 762 Hz. The arrows indicate the decoupling frequencies. It should be noted that the sample in (B) and (C) contained two additional portions (each 0.5 mg) PPIase from two previous experiments.

4. DISCUSSION

The efficiency of catalysis must depend on the isomerase accessibility to the particular proline peptide bond. Lin et al. [15] have studied the effect PPIase has on the slow refolding reactions in seven different proteins. PPIase was found to catalyze the slow refolding step for only the smallest of these proteins (RNase T_1 and cytochrome C). For these two proteins – and for other proteins previously studied [11–14] – the increased rate of refolding caused by the addition of PPIase

was, however, more than 100 times smaller than observed with sterically accessible oligopeptides [7,10]. The relative amounts of PPIase needed to give a 2–7-fold increase in the *cis-trans* isomerization rates are much larger (10–80 mol%) for proteins than for the oligopeptides (a few mol%) [7,10–15]. Lin et al. [15] also observed that PPIase weakly catalyzed the refolding of RNase A in 2.3 M urea while no rate enhancement could be detected in 0.2 M urea. The flexibility and solvent accessibility of the linker loop in calbindin D_{9k} is characterized by the following experimental findings: (i) the rate of interconversion between the *cis* and *trans* conformers at the Gly-42-Pro-43 amide bond is close to that observed for similar amide bonds in small model peptides [6] and *N*-dimethyl substituted amides [24]. (ii) The rate of backbone amide proton exchange with protons in solvent water for the amino acid residues 41–49 are among the highest in the calbindin molecule ([2] and Linse, S., unpublished). (iii) *cis/trans* interconversion at the Gly-42-Pro-43 amide bond is accompanied by very localized changes in tertiary structure and does not affect the global structure of the protein [5].

The finding that the Pro-43 *cis-trans* exchange rate in the solvent-accessible and flexible loop of folded calbindin is unaffected by the addition of PPIase indicates that very special spatial requirements, due to the size of the necessary interaction region, must be fulfilled in order for PPIase to exert its catalytic activity. According to accepted theories of enzyme catalysis PPIase should function by preferentially stabilizing the transition state of the *cis-trans* isomerization process. The steric requirements for the formation of such an interaction complex may not generally be met in folded or partially folded proteins.

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