Pyridoxal phosphate binding to wild type, W330F, and C298S mutants of *Escherichia coli* apotryptophanase: unraveling the cold inactivation

Tali Erez[a], Robert S. Phillips[b], Abraham H. Parola[a,*]

[a]*Department of Chemistry, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84 105, Israel*
[b]*Departments of Chemistry and of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA*

Received 28 June 1998; revised version received 13 July 1998

Abstract The mechanism of pyridoxal phosphate (PLP) binding to apotryptophanase was investigated using stopped-flow kinetics with wild type (WT), W330F and C298S mutants. Based on the dependence of the rate constants on PLP concentrations for the fast and slow phases detected, two mechanistic schemes were proposed. For the WT and C298S mutant, the slow process is due to an isomerization of the aldimine complex after its formation, and not to the binding to an alternative conformation of the apoenzyme, which is the case proposed for the W330F mutant. It is suggested that during the cold inactivation process a conformational change precedes the aldimine bond cleavage. For the W330F apotryptophanase, another conformational change occurs subsequent to the aldimine bond cleavage.

Key words: Tryptophanase; Pyridoxal phosphate; Cold inactivation; Site-directed mutagenesis; Stopped-flow kinetics; Protein conformation and quaternary structure

1. Introduction

Tryptophanase (tryptophan indole-lyase, EC 4.1.99.1) is a widely distributed bacterial pyridoxal phosphate (PLP)-dependent enzyme that catalyzes α,β-elimination and β-replacement reactions of L-tryptophan and of a variety of other β-substituted L-amino acids [1,2]. It consists of four identical 52 kDa monomers. Each monomer contains one molecule of PLP, which forms an aldimine bond with Lys-270; due to this aldimine, Tnase exhibits pH-dependent absorption and CD spectra with maxima at 420 and 337 nm [1–4]. Tnase requires certain monovalent cations (K^+, NH_4^+, Tl^+) for its activity and for tight PLP binding [5–7]. The X-ray structure of tryptophanase from *Proteus vulgaris* was recently reported [8].

Based on analytical ultracentrifugation apo-Tnase, in the presence of either 0.1 M KCl or 0.1 M NaCl and at low temperature (5°C) undergoes a concentration-dependent dissociation into dimers [1]. At 10 mg/ml, however, apo-Tnase exists mainly in the tetrameric form [1].

Studies of the effect of cooling on tryptophanase have shown that WT-Tnase and W330F-Tnase mutant undergo a reversible cold inactivation, yet to a different degree [9]. Rewarming of the cold-adapted enzyme leads to restoration of 80% of the original activity ($t_{1/2}$ = 23 min) and to the reappearance of the initial spectra ($t_{1/2}$ = 14 min) and the tetrameric structure. The C298S mutant form exhibited a similar cold inactivation, yet a more facile one when compared to that observed with the WT form [10]. It was suggested that cooling of the enzyme favors its conversion into an inactive conformation that releases PLP [9]. We have examined the binding of PLP to the apo- form of the WT, W330F and C298S mutant forms of Tnase using single-wavelength stopped-flow kinetics to clarify the mechanism of the cold induced inactivation.

2. Materials and methods

2.1. Chemicals

S-(*o*-Nitrophenyl)-L-cysteine (SOPC) was synthesized as described in [11]. PLP, Tricine and 2-mercaptoethanol were purchased from Sigma.

2.2. Preparation of enzymes

WT-Tnase was isolated from cells of *Escherichia coli* SVS 370 containing the *tnaA* gene on plasmids by the procedure described in [10,12]. Additional purification was achieved by chromatography on DEAE-Sephadex A-50 as described in [13]. W330F-Tnase and C298S-Tnase were isolated in the same way from cells of *E. coli* SVS 370 containing the *tnaA* gene with a respective site-directed mutation. Apoenzymes were prepared by overnight dialysis of the holoenzyme in cold against 0.25 M sodium phosphate buffer containing 0.1 M L-alanine, pH 7.0 [3,14]. The protein concentration was determined by measurement of absorbance at 278 nm, taking $A^{1\%}$ values of 9.19, 7.64 and 7.95 for holo-WT and W330F-Tnases and apo-WT-Tnase, respectively [10,12]. Enzymatic activity was measured by the spectrophotometric method using a chromogenic substrate analog, SOPC, as described in [15]. The enzyme preparations had a specific activity of 45–50 μmol min^{-1} mg^{-1} at 25°C and pH 8.0.

2.3. Stopped-flow studies

All experiments were performed in 50 mM Tricine-KOH buffer, pH 7.5, containing 100 mM KCl, 2 mM EDTA and 5 mM 2-mercaptoethanol. The concentrated solution of the apoenzyme (20 mg/ml) was incubated at 2°C overnight. At this relatively high concentration no dissociation into dimers occurs [1]. Association rate constants for PLP combining with WT or mutant apo-forms were measured under pseudo first order conditions. Shortly prior to measurement (the time delay between the dilution and the initiation of data collection was less than 1 min), the apoenzyme solutions (20 mg/ml) were diluted with cold (2°C) buffer, and measured at 20 μM (1 mg/ml) while PLP was in at least 4-fold excess. No dissociation into dimers occurs within this time period [1,9]. While the PLP solutions were preincubated for 10 min at 30°C, the apoenzyme solutions at 2°C have been injected instantaneously. Equal volumes of these two solutions, 100 μl, were injected. Single-wavelength stopped-flow kinetic measurements were performed at 30°C, with the temperature controlled by the circulating water bath (±0.5°C). Stopped-flow kinetic data were obtained with an instrument from Kinetic Instruments Inc., using hardware and software provided by On-Line Instrument System Inc. (OLIS), as described previously [16]. The dead time for this instrument is 1 ms. The enzyme solutions were mixed with various concentrations of PLP, and the increase in absorbance at 420 nm was followed; the optical path was 20 mm. Typically, 512 data points were collected over a period of several minutes. Exponential absorbance increases of about

*Corresponding author. Fax: (972) (7) 6472943.
E-mail: aparola@bgumail.bgu.ac.il

Abbreviations: Tnase, tryptophanase; WT-Tnase, wild type Tnase; PLP, pyridoxal 5′-phosphate; Tricine, *N*-[2-hydroxy-1,1-bis (hydroxymethyl)-ethyl]-glycine

PII: S0014-5793(98)00931-4

0.05 0.1 were observed, with noise levels of ±0.001 *A*. The rate constants were evaluated by exponential fitting using the LMFIT program (Levenberg-Marquart algorithm) provided by OLIS. The validity of the fitting was evaluated by inspection of residuals, standard deviation or the Durbin-Watson parameters [17]. Typically, rate constants were estimated to a standard deviation of less than 5%. As conducted, this is a non-equilibrium T-jump experiment, followed kinetically by the stopped-flow technique. The time delay before the T-jump experiment is less than 1 min, which is short relative to the time for reactivation of cold-inactivated apotryptophanase ($t_{1/2}$ = 23 min).

3. Results and discussion

The progress curve of the reaction of the apoenzymes with PLP at 420 nm is biphasic and fits well to the sum of two exponential relaxations. Attempts to fit the data to a single pseudo first order or second order model were unsuccessful.

For the W330F mutant Tnase, the observed rate constants for the fast and slow phases (k_{obs}) increased in Michaelis-Menten manner with increasing [PLP] as shown in Fig. 1. If PLP binding occurs with a simple two step mechanism, assuming independent binding [5], as shown in Scheme 1,

$$E + PLP \underset{}{\overset{K_1}{\rightleftharpoons}} EPLP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EPLP_1$$

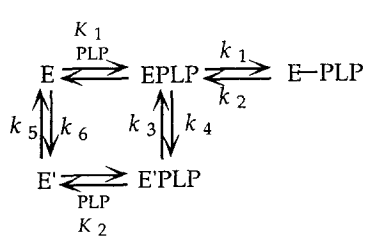
Scheme 1.

then the observed rate constant should increase with increasing [PLP] according to the following relationship.

$$k_{obs} = \frac{k_1 \times [PLP]}{K_1 + [PLP]} + k_2$$

where k_1 and k_2 represent the forward and reverse rate constant, and K_1 is the dissociation constant of the initial binary complex, EPLP. Since the fitting resulted in a *Y*-intercept value that is zero within experimental error, $k_2 \cong 0$, consistent with the results of Hogberg-Raibaud [5]. The resultant parameters for the fast phase are $k_1 = 1.3 \times 10^{-1}$ s^{-1} and $K_1 = 4.5 \times 10^{-1}$ mM (Table 1). The hyperbolic dependence of the observed rate constants for the slow phase on [PLP] suggests the existence of a second conformation that can bind PLP in a mechanism like that shown above. The resultant parameters from fitting the data for the slow phase in the equation are $k_{max} = (1.8 \pm 0.2) \times 10^{-2}$ s^{-1} and $K_{app} = (1.5 \pm 0.5) \times 10^{-1}$ mM.

These data may be consistent with a mechanism involving two different conformations of the enzyme (E and E′) both of which can bind PLP. The first step is the rapid formation of the non-covalent complex (EPLP), which is followed by the slow formation of the internal PLP-lysine aldimine bond (E–PLP), as shown in Scheme 2. E′PLP does not directly convert to E–PLP in a single step. Instead, a conformational change precedes the aldimine bond formation. Judging from the relative amplitudes of the absorbance changes at high PLP concentration, we estimate that E′ represents about 65% of the population of cold inactivated W330F Tnase. This could not be caused by pre-equilibration of dimers and tetramers prior to the T-jump, since the expected percentage of dimers should be less than 5%, as was discussed above comparing the experimental setup time to the $t_{1/2}$ for dimer-tetramer equilibration. The direct interconversion of E and E′ must be slower than the interconversion of EPLP and E′PLP ($k_3+k_4 \gg k_5+k_6$) to allow the observation of the two processes.



Scheme 2.

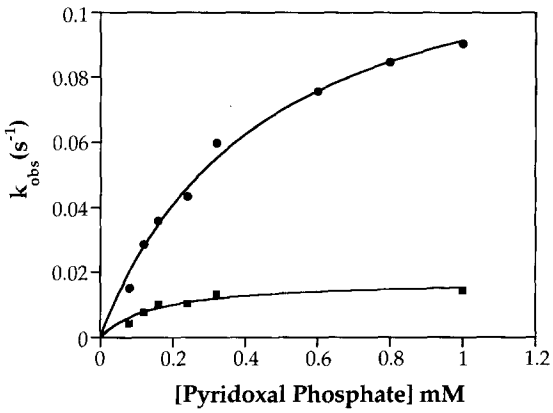


Fig. 1. The observed rate constants for the association of W330F apoenzyme with PLP. Two phases were detected. The reactions were performed at 30°C in 50 mM Tricine-KOH buffer, pH 7.5, containing 100 mM KCl.

In the case of the WT enzyme and the C298S mutant enzymes, the PLP binding reaction also exhibits biphasic kinetics, but in contrast to the results obtained with the W330F mutant the rate constant of the slow process is unaffected by PLP concentration, as shown in Figs. 2 and 3. The hyperbolic dependence of the first phase on [PLP] implies a non-covalent complex similar to that seen for W330F. Thus, the average values of the observed rate constant are $k_{obs} = (4.4 \pm 0.9) \times 10^{-3}$ s^{-1} and $k_{obs} = (6.1 \pm 0.3) \times 10^{-3}$ s^{-1} for the wild type and the C298S mutant, respectively. The kinetic parameters for the fast phase are shown in Table 1. The similarity in both parameters among the three types of enzyme examined, suggests that the fast process is insensitive to the mutations.

$$E \overset{\overset{K_1}{PLP}}{\rightleftharpoons} EPLP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{-}PLP \rightleftharpoons (E{-}PLP)_1$$

Scheme 3.

Table 1
Kinetic constants for the reactions of wild type, W330F and C298S apoenzymes with PLP

Tnase	k_1 (s^{-1})	K_1 (mM)
WT	$(1.7 \pm 0.1) \times 10^{-1}$	$(6.5 \pm 1.0) \times 10^{-1}$
W330F	$(1.3 \pm 0.1) \times 10^{-1}$	$(4.5 \pm 0.7) \times 10^{-1}$
C298S	$(6.9 \pm 0.7) \times 10^{-2}$	$(6.4 \pm 1.2) \times 10^{-1}$

The simplest mechanism accounting for the PLP binding to the apoform of WT and C298S mutant is given in Scheme 3 where E is the apoenzyme, EPLP is a non-covalent complex, E–PLP is the internal PLP-lysine aldimine and $(E\text{–}PLP)_1$ is an isomerized aldimine. Since the slow first order process for WT and C298S Tnase is zero order in PLP, it is not coupled directly to a PLP binding step. This proposed scheme implies that the slow process is due to an isomerization of the aldimine complex after its formation, and not to the binding to an alternate conformation of the apoenzyme, which is the case proposed for the W330F mutant.

Our data do not conform to an anticooperative sequential model for binding of PLP to either WT or W330F, which would require a non-hyperbolic binding profile for PLP [5]. A rate-limiting association of dimers to tetramers, which could perhaps be invoked for W330F (for which we do not have ultracentrifugation data), would predict a decrease in apparent rate constant with [PLP] [18], which is not observed. Neither could a model in which preassociation of PLP exclusively with dimeric apoenzyme, followed by tetramerization of the dimeric holoenzyme explain the biphasic nature of the PLP binding process, as shown in Fig. 1. Moreover, as indicated above, the time course of the experimental procedures is too short to allow for pre-equilibration of dimers and tetramers, which leads us to suggest the involvement of two different conformations for W330F, i.e. E and E′, as the most likely explanation for the data. Our kinetic data for W330F is compatible with the existence of two different PLP binding species without the ability to distinguish between two different conformers or two different oligomeric states (i.e. dimer and tetramer) or both. However, the latter model would require that W330F be predominantly in the dimeric form even at 20 mg/ml.

Based on the microscopic reversibility principle, we now provide evidence to our suggestion that during the cold inactivation process a conformational change precedes the aldimine bond cleavage [9], as shown for WT and C298S mutant Tnases in Scheme 3. C298S, which showed the same activity as WT, served as a positive control, as expected for a mutation at the active site rather than at a tetramer-dimer recognition surface. Another conformational change, detectable only for the W330F mutant, occurs subsequent to the aldimine bond cleavage (Scheme 2). This relatively facile conformational change would be expected particularly for the W330F mutant Tnase, since the replacement of Trp by Phe leads to a smaller hydrophobic surface [12], presumably resulting in an increased flexibility. Such flexibility may account for the conformational change encountered (Scheme 2). However, in C298S, the change from Cys to Ser, both of which are polar residues, is a more isosteric replacement when compared with that in the W330F mutant [10]. Accordingly, the mutation in C298S does not provide the flexibility required for the conformational change observed with the W330F mutant. Thus, not surprisingly, the C298S mutant enzyme is similar to the WT enzyme. Upon cooling the W330F mutant, the E′PLP complex in Scheme 2 is favored, shifting the equilibrium away from EPLP and that in turn, results in the breakage of the aldimine bond in E–PLP. In both WT and C298S mutant tryptophanase, k_4 and k_6 in Scheme 2 approach the value of zero, leading to negligible concentration of the E′ and E′PLP forms. Thus, Scheme 3 describes the stepwise process encountered with the latter.

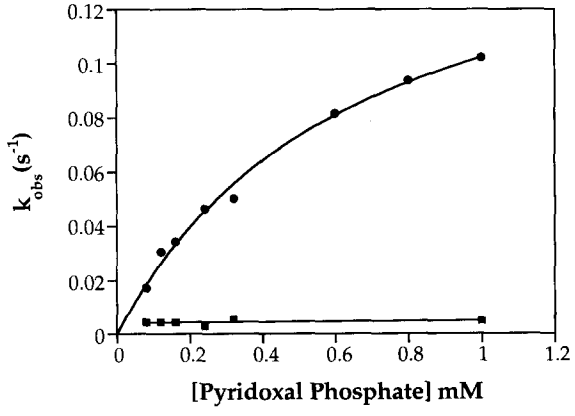


Fig. 2. The observed rate constants for the association of wild type apoenzyme with PLP. Two phases were detected. The reactions were performed at 30°C in 50 mM Tricine-KOH buffer, pH 7.5, containing 100 mM KCl.

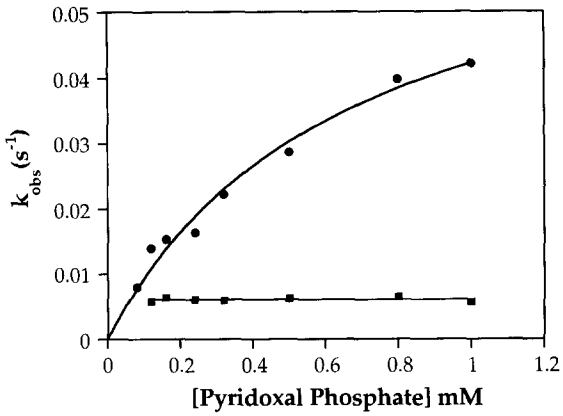


Fig. 3. The observed rate constants for the association of C298S apoenzyme with PLP. Two phases were detected. The reactions were performed at 30°C in 50 mM Tricine-KOH buffer, pH 7.5, containing 100 mM KCl.

In conclusion, based on the proposed mechanism for PLP binding to the WT and C298S apo-Tnases it is suggested that during the cold inactivation process a conformational change ($(E\text{–}PLP)_1$ to E–PLP in Scheme 3) precedes the aldimine bond cleavage (E–PLP to EPLP, in Scheme 3). Furthermore, based on the proposed mechanism for PLP binding to W330F apotryptophanase, we suggest that during its relatively more facile cold inactivation process, an additional conformational change occurs subsequent to the aldimine bond cleavage (i.e., EPLP to E′PLP in Scheme 2). Following PLP dissociation, the ensuing apo enzyme dissociates into dimers [9].

Acknowledgements: This work was partially supported by the James Franck Center for Laser-Matter Interaction, by the Office of Naval Research of the USA (Grants N00014-89-J-1625 and NOOO14-96-1-80) and by the Ministry of Science and Arts of Israel (Grant 86387) to A.H.P.

References

[1] Snell, E.E. (1975) Adv. Enzymol. 42, 287–333.
[2] Torchinsky, Yu.M. and Kawata, Ya. (1994) in: Molecular Aspects of Enzyme Catalysis (Fukui, T. and Soda, K., Eds.), pp. 165–190, Kodansha, Tokyo; VCH, Weinheim.
[3] Metzler, C.M., Viswanath, R. and Metzler, D.E. (1991) J. Biol. Chem. 266, 9374–9381.

[4] Ben-Kasus, T., Markel, A., Gdalevsky, G.Y., Torchinsky, Yu.M., Phillips, R.S. and Parola, A.H. (1996) Biochim. Biophys. Acta 1294, 147–152.
[5] Högberg-Raibaud, A., Raibaud, O. and Goldberg, M.E. (1975) J. Biol. Chem. 250, 3352–3358.
[6] Toraya, T., Nihira, T. and Fukui, S. (1976) Eur. J. Biochem. 69, 411–419.
[7] Suelter, C.H. and Snell, E.E. (1977) J. Biol. Chem. 252, 1852–1857.
[8] Isupov, M., Antson, A.A., Dodson, E.J., Dodson, G.G., Dementieva, I.S., Zakomirdina, L.N., Wilson, K.S., Dauter, Z., Lebedev, A.A. and Harutyunyan, E.H. (1998) J. Mol. Biol. 276, 603–623.
[9] Erez, T., Gdalevsky, G.Ya., Phillips, R.S., Torchinsky, Y.M. and Parola, A.H. (1998) Biochim. Biophys. Acta (in press).
[10] Phillips, R.S. and Gollnick, P.D. (1989) J. Biol. Chem. 264, 10627–10632.
[11] Behbahani-Nejad, I., Dye, J.L. and Suelter, C.H. (1987) Methods Enzymol. 142, 414–422.
[12] Phillips, R.S. and Gollnick, P.D. (1990) FEBS Lett. 268, 213–216.
[13] Honda, T. and Tokushige, M. (1986) J. Biochem. 100, 679–685.
[14] Torchinsky, Y.M., Sakharova, I.S. and Zakomirdina, L.N. (1987) in: Biochemistry of Vitamin B6 (Korpela, T. and Christen, P., Eds.), pp. 233–237, Birkhäuser, Basel.
[15] Suelter, C.H., Wang, J. and Snell, E.E. (1976) FEBS Lett. 66, 230–232.
[16] Phillips, R.S. (1989) J. Am. Chem. Soc. 111, 727–730.
[17] Durbin, J. and Watson, G.S. (1970) Biometrika 37, 409–414.
[18] Chen, H. and Phillips, R.S. (1993) Biochemistry 32, 11591–11599.