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Mechanism of biexponential inactivation of organophosphate hydrolase by 1,10-phenantroline. A kinetic and second derivative UV spectral study

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Abstract

Gentle thermoinactivation of cobalt(II)-dependent organophosphate hydrolase isolated from E. coli DH5α at 25°C and pH 9.0 brings about (i) biexponential loss of the enzymatic activity, which is believed to be due to consecutive removal of two metal ions from the enzyme active site by 1,10-phenantroline (phen) and (ii) the rate law changeover. The rate of both steps becomes independent of [phen] and the corresponding rate constants k_1 and k_2 equal $(4.6 \pm 0.7) \times 10^{-3}$ and $(1.5 \pm 0.2) \times 10^{-4}$ s^{-1}, respectively. The changeover was followed by measuring the second derivative UV spectra of the same preparation of the enzyme and changes in λ_{max} are indicative of its marginal unfolding. Likely, the unfolding distorts the favorable tetrahedral coordinative environment around metals thus facilitating their dissociation.

Keywords: Organophosphate hydrolase; Thermoinactivation; Kinetics; Second derivative UV spectra; Mechanism

1. Introduction

Much interest is currently associated with the organophosphate hydrolase (OPH) metalloenzyme which is thought of high potency in environmental chemistry [1–4] and in decontamination of chemical warfare agents [5]. It is a small metal-dependent enzyme of the molecular mass 39 kDa initially isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551 [6–9]. Its specific substrates are phosphorous pesticides such as parathion, paraoxon, cyanophos and coumaphos, as well as related chemical weapons [3,10]. These are converted by OPH as shown in Eq. (1) by the example of paraoxon.

$$(EtO)_2P(O)OC_6H_4NO_2\text{-}p + H_2O \rightarrow (EtO)_2P(O)OH + HOC_6H_4NO_2\text{-}p \quad (1)$$

There is evidence [11–13] supported by the X-ray structural study of the apoenzyme [14] for the coordinative environment of two metal ions in OPH. Both metals have a tetrahedral coordination. The first is surrounded by three histidine imidazoles, the fourth site being occupied by the crucial for catalysis aqua ligand. The second metal is coordinated by two imidazoles and two oxygen atoms of other amino acid residues the nature of which has not unequivocally been determined. Despite different coordinative envi-

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ronment of two centers, a previous kinetic study of the metal dissociation induced by 2,2′-bipyridine (bpy) or 1,10-phenantroline (phen) did not reveal different reactivity of the metal ions, and the second-order kinetics was observed [9]. Our interests in the field of bioinorganic chemistry and organometallic biochemistry [15] spread on various aspects of functioning of metal ions in the active sites of enzymes and mechanisms of metal removal from metalloenzymes. Therefore, we undertook a detailed kinetic study of the ligand-induced inactivation of OPH. The data obtained showed that a biexponential metal removal, which was not reported before, could be observed under certain conditions and such a behavior is probably accompanied by the associative to dissociative mechanistic changeover.

2. Experimental

2.1. Materials

The source of the enzyme, its preparation and characterization is described in detail elsewhere [9]. The culture was grown in the presence of 1×10^{-3} M $CoCl_2$ to afford the enzyme preparation with two Co^{II} ions in the active site. The enzymatic activity was assayed according to the standard procedure adopted for OPH [13]. Paraoxon (diethyl-*p*-nitrophenyl phosphate) was purchased from Sigma and purified by treating its solution in CH_2Cl_2 with glycine buffer (pH 9). The organic layer was then dried with anhydrous $CaCl_2$, and the solvent was removed in vacuo. Phen and bpy were Chemapol and Fluka reagents, respectively. Glycine for buffer solutions was obtained from Reanal.

2.2. Procedures

A typical procedure for inactivation of OPH in the presence of bpy and phen was as follows. The enzyme solution (2.55×10^{-7} M) in the 0.1 M glycine buffer (pH 9.0) was thermostated in a test tube at 25.0 ± 0.1°C. The first aliquot (0.01 ml) was withdrawn prior to addition of bpy or phen, diluted 200-fold by the buffer containing 5×10^{-6} M paraoxon, and an increase in absorbance at 400 nm due to the enzymatic production of *p*-nitrophenolate ($\epsilon = 17\,000$ M^{-1} cm^{-1}) was then monitored. The slope of linear absorbance versus time plot, V^0_{obs} (M s^{-1}) was taken as 100% of the enzymatic activity. Bpy or phen was then added to the enzyme solution. Aliquots were withdrawn after certain time intervals and treated as described above. The absorbance versus time straight lines were observed at different fixed concentrations of bpy or phen. Calculation of V_{obs} was based on initial branches of these lines so that the inactivation of OPH occurring in the UV–vis. cell after the dilution was neglected. The data were treated by plotting $\ln(V^t_{obs}/V^0_{obs})$ against time in the case of monoexponential inactivation to obtain the pseudo-first order rate constants k_{obs}. Biphasic kinetics was fitted to the equation $V^t_{obs} = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$ using a nonlinear regression analysis.

Inactivation of the enzyme in solution at 25°C without phen was studied at higher [OPH] (1.3×10^{-5} M) in order to measure the second derivative UV spectra. To assay both the enzymatic activity and the kinetics of phen-induced inactivation, aliquots were withdrawn after certain time intervals, diluted with the glycine buffer to achieve [OPH] = 2.55×10^{-7} M. Such solutions were treated as above to follow the kinetics of thermal and ligand-induced inactivation.

Spectrophotometric measurements were carried out on a Hitachi 150-20 instrument. The second derivative UV spectra were obtained on a Shimadzu 265 FW spectrophotometer. Denaturing polyacrylamide gel electrophoresis was carried out by the method of Chan et al. [16].

3. Results and discussion

3.1. Preliminary observations

As mentioned, the inactivation of OPH by bpy, phen, and EDTA obeys the second-order

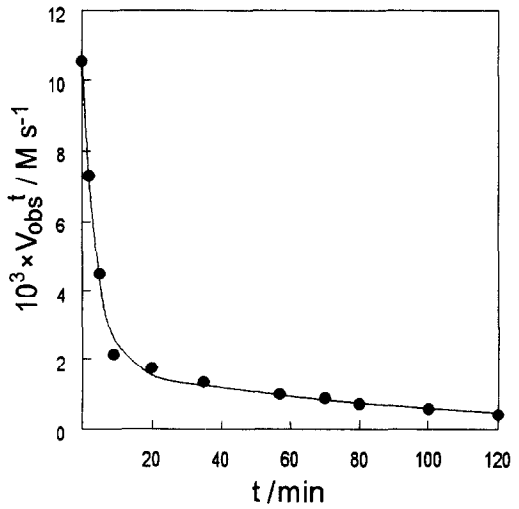

Fig. 1. Loss of activity of OPH in the presence of phen, 25°C, pH 9.0, 0.1 M glycine, [OPH] = 2.55×10^{-7} M, [phen] = 2.04×10^{-5} M.

kinetics, the reaction being first-order in enzyme and ligand. Our experiments with the Co^{II}-dependent OPH confirmed the results obtained previously [9]. In particular, it was found that the pseudo-first-order rate constants for inactivation k_{obs} evaluated from the monoexponential activity versus time plots were proportional to the concentration of bpy, the second-order rate constant being 5.3 M^{-1} s^{-1}.

When the stock solution of the enzyme was repeatedly removed from a refrigerator where it was kept at 4°C, we began to observe a reproducible inflection point on the activity versus time plots. A typical example is shown in Fig. 1. It is clear that instead of the monoexponential loss of activity observed for the native preparation, there is a biexponential phen-induced inactivation of an 'older' enzyme. Since all runs were made in a large excess of phen with respect to OPH, the stoichiometry of the reaction resulting in complete loss in activity is given by Eq. (2)

$$E(M^{II},M^{II}) + 6\text{phen} \rightarrow E(\square,\square) + 2M(\text{phen})_3^{2+} \quad (2)$$

where □ indicates an empty metal binding site. The formation of $Co(phen)_3^{2+}$ in excess of phen is well documented [17,18]. The biexponential kinetics could arise, in principle, from either the consecutive removal of two metal ions or generation of the second isoenzyme. The latter possibility was ruled out since the electrophoregrams of 'biexponential' and native OPH were identical. Therefore, we conclude that the prolonged keeping of OPH in solution even at 25°C generates the kinetic non-equivalence of the two metal ions which manifests itself in the biexponential phen-induced inactivation (Fig. 1).

3.2. Attaining biexponential kinetics on mild inactivation

To trace the effects responsible for changing the kinetic behavior of OPH, a *mild* inactivation of OPH was carried out. The enzyme was kept in solution at 25°C and we measured (i) residual enzymatic activity, (ii) kinetics of inactivation by phen with special emphasis for the monoexponential → biexponential changeover, and (iii) the second derivative UV spectrum of OPH [19–21]. The latter deserves a comment. The second derivative UV spectroscopy, being non invasive technique, gives information on relative protein unfoldness [19]. The probes are the tryptophane and tyrosine residues. The unfolding affects the feasibility of water molecules to the aromatic amino acid residues and, as a result, there is a shift of the position of absorbance maximum in the second derivative UV spectrum. The estimated maximum for a completely folded model protein, in which the corresponding residues are fully buried inside the protein globule, is at 291.8 nm. When the protein is completely unfolded and the residues are exposed to water, the maximum moves to 288.8 nm [21]. These estimations hold for the tryptophane residues. Thus, the location of the second derivative maximum reflects the relative protein unfoldness. OPH is very appropriate for such a study, since it has 22 tryptophane and 7 tyrosine residues [7,22]. The tryptophanes are diagnostic, since the extinction coefficient for tryptophane

is by a factor of 3 higher than that for tyrosine in the second derivative UV spectra [21].

There is a gradual loss in the activity on mild inactivation (Fig. 2a) which follows a first-order kinetics with the k_{obs} of (0.26 ± 0.06) days^{-1}. The initial rate of phen-induced inactivation increases with time too. The biexponential kinetics appears after ca. 20 days of the mild thermoinactivation. The kinetic changes were compared with the second derivative UV spectra of the *same* enzyme preparation. As seen from Fig. 2b, λ_{max} levels off, but the drift is only 12% of the highest to be expected [20]. The shift from 291.2 to 290.8 nm, which is believed to be meaningful for the *second derivative spectra* [23,24], corresponds to a 5% increase in the exposure of tryptophane residues [21]. Hence, the mild thermoinactivation brings about only marginal unfolding which, however, affects the kinetics of enzyme demetalation.

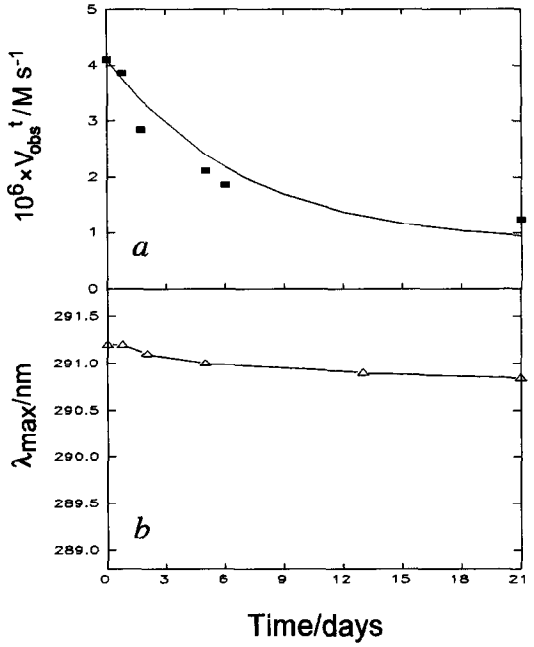

Fig. 2. (a) Loss of activity of OPH on mild inactivation and (b) the shift of the second derivative maximum of OPH with time (25°C, pH 9.0, 0.1 M HEPES, [OPH] = 1.3×10^{-5} M).

3.3. Kinetics of biexponential inactivation by phen

The biexponential inactivation was studied at different [phen] to evaluate its effect on k_1 and k_2. The data are shown in Fig. 3 where k_1 and k_2 refer to the faster first and the slower second step, respectively. As seen, instead of the first-order dependence in phen observed for the native enzyme, there is a zero order for mildly inactivated OPH. At 25°C, the rate constants k_1 and k_2 are $(4.6 \pm 0.7) \times 10^{-3}$ and $(1.5 \pm 0.2) \times 10^{-4}$ s^{-1}, respectively.

3.4. Mechanistic considerations

To our knowledge, this is the first report on this particular kinetic changeover for inactivated metalloenzyme. It should be pointed out, however, that mechanisms of metal removal from metalloenzymes is a poorly investigated area [25–27]. We showed here that mild inactivation may change the rate laws for native and inactivated OPH. Independence of k_1 or k_2 on [phen] is an accord with the dissociative mechanism of demetalation which is also supported by the fact that OPH loses its activity in very dilute solutions. Assuming that the two steps refer to the consecutive removal of two metals, Eqs. (3)–(5) are in agreement with the observed rate law.

$$E(M^{II},X) \rightleftarrows E(\square,X) + M^{II} \; k_+;k_- \qquad (3)$$

$$M^{II} + \text{phen} \rightarrow M(\text{phen})^{2+} \; k_{phen} \qquad (4)$$

$$M(\text{phen})^{2+} + 2\text{phen} \rightarrow M(\text{phen})_3^{2+} \text{ fast} \qquad (5)$$

where X stands for either M^{II} or $\square$. The second-order rate constant k_{phen} is 3.2×10^5 M^{-1} s^{-1} [17]. At [phen] used $(2.04 \times 10^{-5}$ M) the corresponding first-order rate constant equals 6.45 s^{-1}, and it is by a factor of ca. 10^3 higher than k_1 which, therefore, must be treated as rate limiting and irreversible.

There were speculations on a role played by two metal ions in the OPH catalysis. The first metal ion is believed to be crucial for catalysis while the second ion plays a supportive role [13]. The kinetic data obtained here conform the mechanism proposed earlier. As a rule, the in-

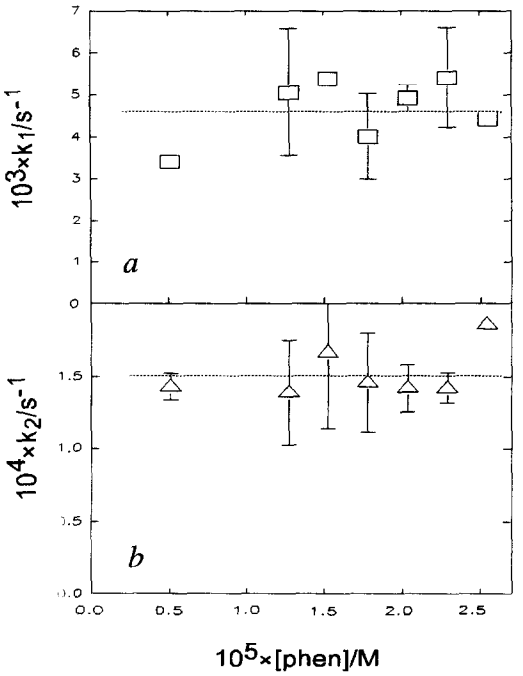

Fig. 3. Rate constants for the first (a) and second step (b) of demetalation of OPH by phen at pH 9.0, 25°C, [OPH] = 2.55×10^{-7} M.

flection point in Fig. 1 corresponds to a 80–85% loss in the total activity. One may thus assume that the dissociation of the first, crucial for catalysis, metal ion provides the major inactivation. At the same time the catalysis, although less efficient, is also possible at the second metal center. Hence, OPH adopts a regulatory mechanism for changing its activity on going from the $E(M^{II},M^{II})$ to the $E(M^{II},\square)$ state.

The origin of the kinetic changeover is worth mentioning. Likely, the tetrahedral coordinative sphere in the native metalloenzyme made of three histidines and water ensures the strongest metal binding. It has been shown for carbonic anhydrase II that, if one of such key histidines is substituted by alanine, cysteine, asparagine or glutamine, the tendency of Zn^{II} to dissociate increases by a factor of 10^5 [26]. In native OPH, the metal binding is strong enough and the second-order kinetics of, presumably associative, demetalation is realized. The mild inactivation induces the partial unfolding and distortion of the optimal coordinative sphere. Consequently, the metal binding weakens and, hence, the fist-order dissociative pathway becomes operative.

4. Conclusion

The second order → first order kinetic changeover in the phen-promoted dissociation of two metal(II) ions from the active site of OPH is induced by its mild inactivation and, hence, slight unfolding. The coordinative sphere around tetrahedral metal ions becomes looser making the phen-independent removal the dominant pathway. The two metals attain kinetically different environment and this accounts for a biexponential kinetics of the ligand-induced inactivation.

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