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Catalytic properties, stability and the structure of the conformational lock in the alkaline phosphatase from *Escherichia coli*

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Abstract

The activity of oligomeric enzymes is sensitive to the formation and dissociation of the interprotein contacts that make up the conformational lock. The mechanism for this is discussed in this article concerning the alkaline phosphatase (AP) from *Escherichia coli*. Study of the AP from various source shows that the thermoinactivation curves, obtained under various conditions, have induction periods that may be ascribed to latent structural changes in the conformational lock. The analysis of kinetic curves has allowed us to calculate the minimum number of denaturation stages in the conformational lock ($n = 3$), i.e., the stable dimer becomes labile and capable of dissociation by the sequential dissociation of two of the three contacts which take part in the conformational lock. Three-dimensional structural analysis of AP from *E. coli* established that the structure of intersubunit contact is formed by three sites: two identical peripheral sites, formed by loop 1–29 and helix 29–34 (H 29–34) of each subunit and one, located near to the active centers of two subunits. Destruction of two contacts does not effect the catalytic activity but opening the third results in the dissociation of dimers into monomers and loss of catalytic activity. Thus, kinetic calculation is correlated with the results of structural analysis. Oligomers with decreased activity and increased stability were found in solutions of *E. coli* AP. The structural possibilities for tetramer formation, based on packing of molecules in protein crystals, are discussed.

Keywords: Alkaline phosphatase; Conformational lock; Stability; Structure; Denaturation; Oligomers; *Escherichia coli*

1. Introduction

The properties of interprotein contacts in oligomeric enzymes and their influence on catalytic activity may be studied and explained by two independent methods—using their structural data and the results of chemical kinetics. In this article, it is shown that results of both methods are in reasonable agreement using alkaline phosphatase (AP, EC 3.1.3.1) as an example. AP from *Escherichia coli* catalyses the hydrolysis of a variety of phosphomonoesters at similar rates; the reaction proceeding through a phosphoenzyme intermediate. The active site region is highly conserved between the *E. coli* and mammalian AP [1]. Our analysis of interprotein contact structure in AP from *E. coli*, based on X-ray data [2] has shown that the

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minimum number of kinetic stages and the number of contact sites within the dimeric subunits are both equal to three. Previously [3], we have shown that the stability of the interprotein contact is probably mostly influenced by residues Gln 410, Glu 411 and Gln 416. Destruction of all of the non-covalent interactions of any of these residues (which may result from substituting the residues) might lead to a significant effect on loop 402–417 and in complete loss of catalytic activity. This loop contains the residue His 412, which is essential for catalysis (co-ordination of metal ion Zn1). Thus, the work [3] offers a structural explanation of the

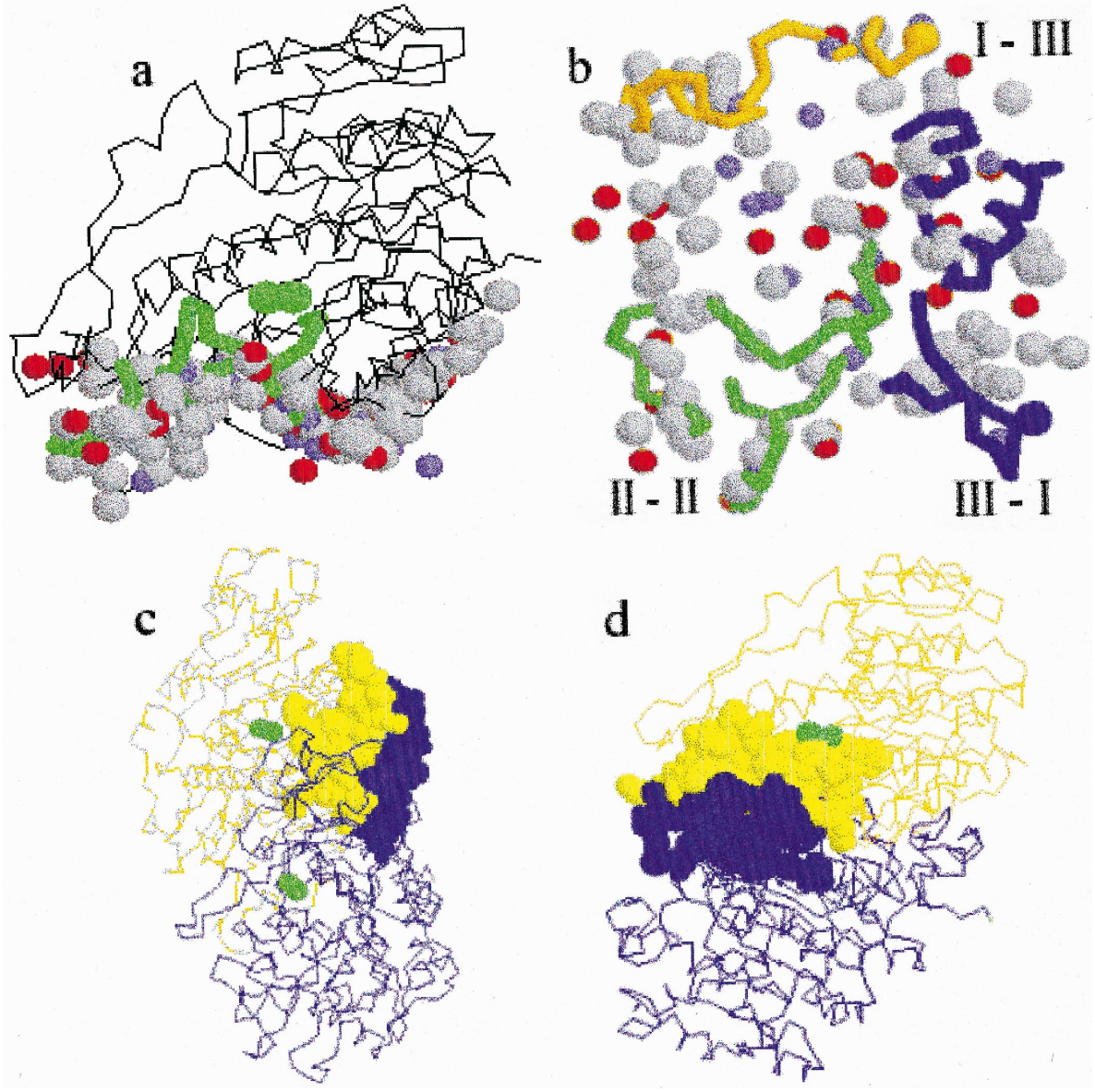

Fig. 1. Model of the dimer of AP from *E. coli* on the basis of X-ray data [1]. (a) Polypeptide chain of one globule (Zn ions—green balls, grey—HPints of interglobular contact, red and blue—electronegative atoms of interglobular HBonds). Green broken line—polypeptide chain of II–II contact. (b) Interglobular contacts I–III and II–II for one globule. (c) Interglobular contact I–III in dimeric enzyme (orange polypeptide chain and yellow balls—globule 'A', Zn ions—green). (d) Interglobular contact II–II affected ligands of Zn ions.

Table 1
Intersubunit contact sites of *E. coli* AP

Contact site	SSEs[a] making the site	SSEs of neighboring subunit, making the complementary part
I–III and III–I	L 1–29	L 85–101
	H 29–34	LSH 422–447
II–II	L 402–417	L 402–417
	LSL 367–386	LSL 367–386

[a]SSE: helix (H), strand (S), loop (or turn or random coil), (L) or a sequential combination of them.

activity loss during the dissociation of AP dimer according to scheme (1).

$$E_2(3), \textit{stable} \leftrightarrow E_2(2) \leftrightarrow E_2(1), \textit{labile} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} 2E_1 \overset{k_d}{\rightarrow} 2E_d \quad (1)$$

where $E_2(3)$ is the native dimer with three intact contacts, $E_2(2)$ has two intact contacts, $E_2(1)$ is the labile dimer, and E_1 is the inactive monomer. E_d is the denatured monomer and k_1, k_{-1} and k_d are rate constants.

Each subunit has been described as having the form of a wedge [4] and is shown in Fig. 1 using the RASMOL program. Secondary structural elements of one of the inclined planes form the interprotein contact and part of the active center. Placement of the subunits may be described roughly by the application of an inversion symmetry operation that includes 180° rotation relative to each other. The topology of the subunit fold corresponds to the α/β class. Despite the similarities in the overall α/β fold with other proteins, AP does not have the characteristic-binding cleft at the carboxyl end of the parallel sheet. However, it has a small 'active pocket' containing three functional metal sites located off the plane of the central 10-stranded sheet. This active pocket is located between the plane of the sheet and two helices on the same side.

Two identical contacts are formed by loop 1–29 (L 1–29) and helix 29–34 (H 29–34) of one subunit, complemented by the SSEs (secondary structure elements) L 85–101 and LSH 422–447 of the other subunit. The SSEs making the sites are given in Table 1 with the properties of all of the contact sites presented in Table 2. Glu 411 forms two 'forked' hydrogen bonds with Thr 381 of the other subunit ('forked' means that the hydrogen of the OH– group in threonine is donated to both oxygen atoms of glutamate) and Gln 416 forms 'bidentate' bonds with the Arg 62, placed on the H 54–66 of the other subunit ('bidentate' means that the oxygen accepts two guanidinium hydrogen atoms from the arginine). Thus, the residue at the active center (His 412) and the intersubunit contact residues (Glu 411 and Gln 416) are adjacent. His 331, His 412 and Asp 327 maintain the position of the catalytic zinc atom Zn1. From these data, we have to conclude that interprotein contact II–II is strongly connected with active center of enzyme and that dissociation of dimer has to destroy the conformation of active center by changing the positions of ligands of both zinc ions.

Contacts III–I and I–III are identical and are placed at the base of the subunits' wedges, contact II–II is inside the dimer near the active centers. The distance between the C-terminus and N-terminus is about 30 Å. Accessibility calculation [1] show that the active center, or better 'pocket', barely accommodates an inorganic phosphate ion and, therefore, the alcoholic or phenolic portion would be in the broad cleft outside the enzyme.

The kinetic data for AP of animal origin (from chicken intestine) and the calculated value of n (number of stages before dissociation, $n \sim 3$–4) have previously been established [4]. However, similar data were not obtained for the

Table 2
Some properties of dimer contact sites in the *E. coli* AP molecule

Contact site	II–II	I–III
Surface, Å^2 (approximate)	400	420
Number of hydrogen bonds (HBonds)	15	12
Number of hydrophobic interaction between two atoms (HPints)	25	40
Number of water molecules per contact	3	8

AP from *E. coli*. In the present work, the thermostabilities of AP of animal and bacterial origin are compared. Investigation of *E. coli* AP thermoinactivation presents extra difficulty, due to the relatively high stability of the *E. coli* dimer, which is stable up to 70°C. The problem is producing kinetic curves with induction periods, which are necessary for calculating the minimal number of intermediate stages that describe the dimer destruction. Therefore, for denaturation, no more than 2.5 M urea was used instead of the 8-M urea solution usually used.

2. Results and discussion

Fig. 2 gives the thermoinactivation curve of AP of animal origin at room temperature under various urea and Mg^{2+} ion concentrations. The kinetic curves at concentrations of urea up to 1 M are of the same type. After 8–10 h, a new equilibrium is established, and the activity values exceed the initial values by 25–30% for the experiments in the absence of Mg^{2+} ions. The occurrence of this maximum is explained by the presence of two oppositely directed processes: dissociation of the less active oligomers (for instance tetramers) to the more active dimers, fixed by a conformational lock, and the partial dissociation of labile dimers into inactive monomers according to scheme (1).

One polar and several hydrophobic patches form the putative region of dimer association. The polar patch (seven atoms) is formed by the side-chain atoms of Glu 8; Lys 73; Gln 350; Glu 354; Lys 357. The residues forming hydrophobic patches (five patches, 26 atoms, main residues are Val 266, Trp 268, Pro 271, Val 284, Pro 288) are mostly to be found within the chain segment comprising of loop, helix, loop (LHL 259–298). Change in conformation of LHL 259–298 could influence the dimer's activity through the disulfide bond Cys 286–Cys 336 interacting with the active site's Zn-binding helix 325–333. As the putative interglobular contacts that form the hydrophobic patches are placed on the contrary side of the dimer molecule, formation of linear associates from several dimers is possible. Possibly, they were not found due to the instability of such complexes.

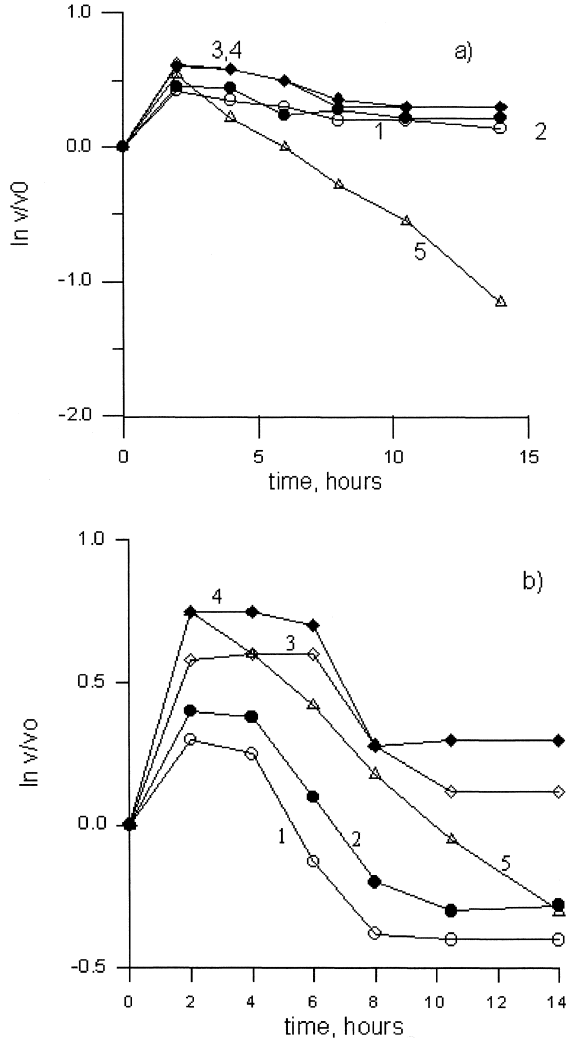

Fig. 2. Kinetics of inactivation of AP from chicken intestine from Sigma at pH 8.5 in 0.05 M Tris–HCl buffer, temperature 20°C and various concentrations of urea: (1) 0; (2) 0.1 M; (3) 0.5 M; (4) 1.0 M; (5) 2.5 M. The enzyme concentration was 3 μg/ml. The AP activity was measured using 6 mM *p*-nitrophenylphosphate as substrate, in 0.05 M Tris–HCl buffer, pH 8.5, containing 1 mM $MgCl_2$. The product (*p*-nitrophenol) was measured spectrophotometrically at 400 nm. (a) In the absence of Mg^{2+} ions; (b) in the presence 1 mM of Mg^{2+}. v, v_0—velocity and initial velocity respectively, v/v_0—relative activity; t—time.

Surface hydrophobic and hydrophilic contacts and protein crystal molecular contacts were examined and analyzed to define the ability of the AP molecule to form oligomers made from more than two subunits (e.g., tetramers). The SSEs making this crystal interaction are on one of the principal faces of the molecular 'wedge', while another principal face of the wedge makes the intersubunit contact of the dimer as described above. The former principal face contains the hydrophobic contacts and 'cores', found previously by molecular surface analysis. These hydrophobic contacts correspond to about 15 interglobular hydrophobic atomic contacts if the criteria for hydrophobic contacts were widened by addition of 0.5 Å. Also two hydrogen bonds were found as part of two 'salt-bridges' between Glu 126 and Lys 127 and vice versa. Thus, tetramers may be formed from two dimers by interaction of their hydrophobic contacts. Tetramer formation of $A_{A(cr)}$ type may influence on activity as follows. Loop 112–131, which makes crystal interaction and probable participates in tetramer formation, is placed in space near Ser 102 of the active center (one HBond to be found between Asp 101 and Gly 118). Another neighbor of the loop is Arg 166, participating in phosphate binding (there is also an HBond between Arg 166 and Asp 101). Clearly, a change in the loop 112–131 conformation can lead to change in enzyme activity.

The position of the maximum on the kinetics curves changes in presence of Mg^{2+} ions (Fig. 2b). If the urea concentration changes within the range 0–1.0 M (curves 1–4), the new equilibrium of the active forms of the enzyme is achieved in 8–10 h, as when Mg^{2+} ions are absent (Fig. 2a), but with different activities that depend on the urea concentration. In the absence of Mg^{2+} ions the new equilibrium depends little on the presence of 0–1.0 M urea. Both curves have a maximum due to the two processes: dissociation of less active oligomers to active dimers and dissociation of active dimers to inactive monomers, which then irreversibly inactivate. The presence of Mg^{2+} ions does not influence on this process when using 2.5 M urea. According to our kinetic model, increasing the urea concentration facilitates the dissociation of the less active tetramer. In the 2.5-M urea, two processes occur simultaneously: dissociation of the less active oligomers, and enzyme inactivation with a first order rate constant of 4.2×10^{-5} s^{-1}.

Fig. 3 gives the thermoinactivation curves of the enzyme at 50°C. These curves do not show a maximum, as the increased temperature causes an accelerated dissociation of oligomers into dimers. The induction period is related to the conformational changes. Because of these, the active stable dimer $E_2(3)$ forms the labile dimer $E_2(1)$ capable of inactivation. According to the data given in Fig. 3 with increasing urea concentration from 0 up to 2.5 M, the rate grows from 5×10^{-5} s^{-1} up to 31×10^{-5} s^{-1} and the period of induction decreases. Calculations of the number of intermediates are possible when the rate of dissociation of the oligomers into active dimers has a higher velocity than the rate of the destruction of the intersubunit contacts in the dimers. This condition is met for *E. coli* AP inactivation. The number of stage (n) is calculated from Eq. (3) proposed in Ref. [5].

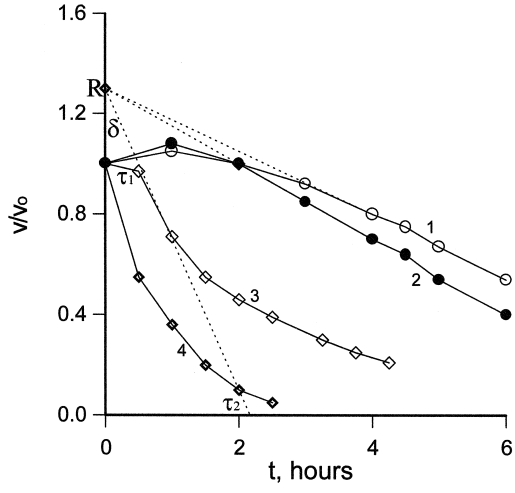

Fig. 3. Kinetics of inactivation of AP from chicken intestine at pH 8.5 and 50°C and various concentrations of urea: (1) 0; (2) 0.1 M; (3) 1 M; (4) 2.5 M (Conditions, see Fig. 2).

For scheme (1), the exact analytical solution gives the value δ that is dependant only on the number of steps before loss of activity (n). To determine this value from the kinetic curves it is convenient to introduce the dimensional ratio of period induction τ_1 and period of inactivation τ_2 shown in Fig. 3.

$$\delta = \frac{\tau_1}{\tau_2 - \tau_1} = R - 1,$$

R is shown in the ordinate of Fig. 3.

For scheme (1), the exact analytical solution gives that value δ is depending only of number of steps before loss of activity n.

$$\delta = e^{-(n-1)}\left[1 + \frac{(n-1)^{n-2}}{(n-2)!} + \sum_{m=1}^{n=2}\left(\frac{(n-1)^m}{m!}\right) - 1\right] \quad (2)$$

However, the exact calculation of n is practically impossible from this expression. For this reason, it was approximated by the empirical equation

$$\delta = \frac{0.13(n-1)}{1 - 0.05n}$$

or

$$n = \frac{0.13 + \delta}{0.13 - 0.05\delta} \quad (3)$$

Such calculation gives only limiting minimal value of n for scheme (1). The error connected with the use of approximate formula (3) instead of precise Eq. (2) is less than 4% when $3 \leq n \leq 11$ and reaches 12% at $n = 2$.

The value of n is about three at concentrations of urea in the range 0–1 M. In the 2.5-M urea, the induction period disappears due to a sharp increase in the rate of inactivation. Fig. 4 shows the thermoinactivation kinetic curves for AP from *E. coli* at room temperature in the presence of the 2.5-M urea (curve 1). Comparison with the appropriate curve for AP from chicken intestine (curve 5 in Fig. 2a), shows that the latter enzyme is less stable. The whole process is finished in 14 days for the chicken enzyme, whereas AP from *E. coli* loses only 60% of its activity in 100 days.

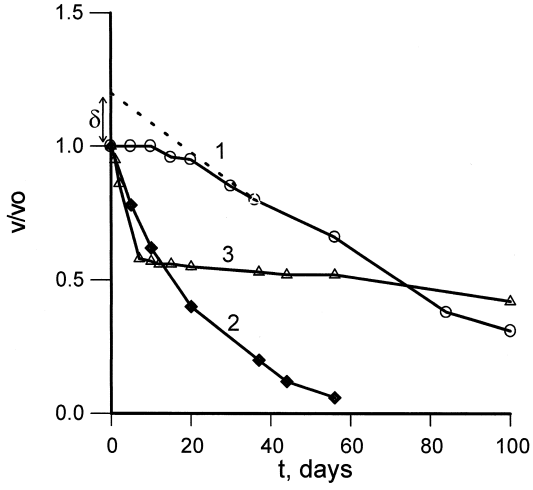

Fig. 4. Kinetics of inactivation of AP from *E. coli* (Sigma) at pH 8.5 and 20°C. (1) In the presence of 2.5 M of urea; (2) in the presence of 2.5 M urea and 1 mM Mg^{2+} ions; (3) in the presence of 1 mM Mg^{2+} ions (Conditions, see Fig. 2).

Two other thermoinactivation curves were obtained in the presence of magnesium and 2.5 M urea. In the presence of Mg^{2+} ions the enzyme sharply loses activity down to 50%, and then the inactivation process slows.

The enzyme from *E. coli* does not require activation by Mg^{2+} ions in contrast to the enzyme from chicken intestine and other enzymes of animal/bird origin. In this case, additional sites may bind the Mg^{2+} ions, but the existence of these sites on the globular surface is under review.

These processes are finished in the first 2–3 days. Any experiment with addition Mg^{2+} ions and urea results in a complete inactivation of *E. coli* enzyme in 60 days. Analysis of the curves in Fig. 2b gives an enzyme activity in the presence of magnesium ions of 0.65 of that of the native form, while the addition of both Mg^{2+} and urea, on the contrary, results in an

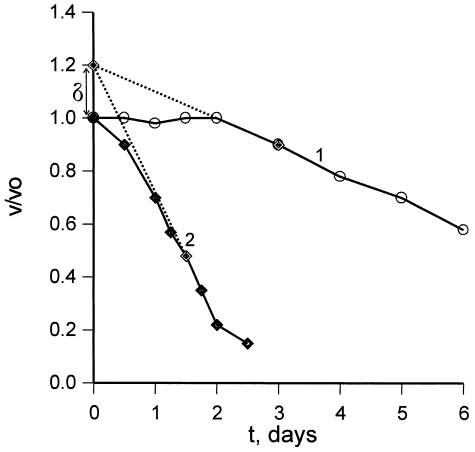

Fig. 5. Kinetics of thermoinactivation of AP from *E. coli* (Sigma) at pH 8.5 in the presence of 2.5 M of a urea at 60°C (1) and 70°C (2).

increase of activity of the enzyme. The inactivation rate decreases in the presence of Mg^{2+} and urea. These data indicate a double influence of Mg^{2+} ion concentration on the stability of AP from animal/bird: on the one hand—destabilization connected with increased Mg^{2+} binding by additional sites, and on the other—increase in dimer stability.

Structural analysis of AP from *E. coli* enzyme has shown that the Asp 51 from the Mg^{2+} co-ordination sphere is close to the intersubunit contact, formed by helix 54–66 including amino acid residues Asp 55, Thr 59 and Arg 62 with Gln 416 of the neighboring subunit. It is possible that in the absence of Mg^{2+}, helix 54–66 moves under the action of the neighboring subunit, as this would result in the slow destruction of the contact site. In the AP from *E. coli* magnesium is strongly bound by the protein molecule. Binding to the protein is less strong in the AP from chicken intestine and for optimum activity, it is necessary to add Mg^{2+} ions. However, excess magnesium inhibits the enzyme. Thermoinactivation curves of AP from *E. coli* (Fig. 5) in the presence of 2.5-M urea at 60°C and 70°C have induction periods depending on temperature. In both cases *n*-value is about 3. Fig. 5 shows the inactivation rates of AP from *E. coli*. In the presence of magnesium ions, the rate increases sharply. As magnesium ions are strongly bound by the enzyme from *E. coli* [6,7], addition of further amounts only increases the binding to the putative additional sites, which accelerates the destruction processes.

Evaluations, based on the number of hydrogen bonds in intersubunit contact of *E. coli* AP give a value for HBond component of dissociation activation energy for the dimer of about 14 kcal mol^{-1} for contact site II–II, (the one connected with the active centers) and 12 kcal mol^{-1} for each intersubunit contact site I–III.

3. Conclusions

As a summary of structural analysis of dimeric molecule of AP we have to conclude that two interprotein contacts (III–I and I–III) may be regarded as independent on of catalytic center. The third contact (II–II) is deeply connected with elements of secondary structure of active center and dissociation of this contact has to destroy the active center. According to these results, the total number of contacts in the conformational lock is three and this is in good agreement with kinetic data, giving the same minimal number of intermediates during the inactivation of AP.

Acknowledgements

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