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Inactivation of jack bean urease by *N*-ethylmaleimide: pH dependence, reversibility and thiols influence

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Abstract
N-Ethylmaleimide (NEM) was studied as an inactivator of jack bean urease at 25 °C in 20 mM phosphate buffer, pHs 6.4, 7.4, and 8.3. The inactivation was investigated by incubation procedure in the absence of a substrate. It was found that NEM acted as a time and concentration dependent inactivator of urease. The dependence of urease residual activity on the incubation time showed that the activity decreased with time until the total loss of enzyme activity. The process followed a pseudo-first-order reaction. A monophasic loss of enzyme activity was observed at pH 7.4 and 8.4, while a biphasic reaction occurred at pH 6.4. Moreover, the alkaline pH promoted the inactivation. The presence of thiol-compounds, such as L-cysteine, glutathione or dithiothreitol (DTT), in the incubation mixture significantly slowed down the rate of inactivation. The interaction test showed that the decrease of inactivation was an effect of NEM-thiol interaction that lowered NEM concentration in the incubation mixture. The reactivation of NEM-blocked urease by DTT application and multidilution did not result in an effective activity regain. The applied DTT reacted with the remaining inactivator and could stop the progress of enzyme activity loss but did not cause the reactivation. This confirmed the irreversibility of inactivation. Similar results obtained at pH 6.4, 7.4 and 8.4 indicated that the mechanism of urease inactivation by NEM was pH-independent. However, the pH value significantly influenced the process rate.

Keywords: *urease, inactivation,* N-*ethylmaleimide, inhibition*

Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea: $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$. The enzyme is widely distributed among plants, algae, fungi and several microorganisms. Common occurrence of urease in the environment enables an efficient decomposition of large quantities of urea as a product of catabolism of nitrogen-containing compounds. On the other side, the products of urea hydrolysis cause the pH increase that negatively effects on human and animal health as well on the environment. Urease serves as a virulence factor in human and animal infections of the urinary and gastrointestinal tracts [1–5]. Moreover, high activity of urease in soil decreases economic use of urea as a fertilizer [6–8]. The use of effective urease inhibitors enables to counteract these negative actions as well give insight into urease structure and catalytic mechanism.

Jack bean urease is a homohexameric molecule. Each subunit contains two nickel ions active site. Spectroscopic investigations indicated that Ni(II) ions are 3.26 Å apart with a coordination environment of the type $Ni(His)_x(N,O)_{5-x}$, with $x = 2$ or 3. This metallocenter is directly involved in binding of substrates and inhibitors [3].

Urease is thiol rich enzyme. Jack bean urease contains in total 15 cysteine residues per subunit. Used of 2,2′-dipyridyl disulfide, 5,5′-dithiobis(2-nitrobenzoic acid) and other thiol modifying agents showed different reactivity and importance of these

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groups. One of them, cysteine-592, $pK_a = 9.15$, is located on the flexible flap covering the active site. Modification of this unique cysteine results in loss of activity. This proved that cysteine-592 plays a significant role in the catalytic activity [3,9–11].

N-ethylmaleimide is commonly used as a titrating agent for determination of protein sulfhydryl groups. NEM was also applied to studying thiols in ureases [11,12]. However there is a little data regarding the details of kinetics and pH influence.

The present study is aimed at elucidation of the pH influence on the kinetics of jack bean urease inactivation by NEM. The process was studied at acid and alkaline pH. The kinetics of inactivation was approached. The influence of thiols and the reversibility of the urease-NEM complex were investigated.

Materials and methods

Materials

The Jack bean urease, Sigma type III of specific activity 22 units/mg protein, was used. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7.0 and 25 °C. N-ethylmaleimide (NEM), L-cysteine (L-cys), dithiothreitol (DTT), glutathione (GSH), were purchased from Sigma. Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade.

Inactivation progress curves

The progress of urease inactivation was studied in 20 mM phosphate buffer, pH 6.4, 7.4 and 8.3, respectively, 1 mM EDTA at 25 °C. The concentrated solution of urease was incubated with the concentrated solution of NEM in the absence of substrate. This mixture was called the incubation mixture. The incubation mixture contained 0.75 mg cm^{-3} of urease and different concentrations of NEM, as specified below:

pH 6.4–1.0, 3.0, 4.0, 5.0 mM
pH 7.4–2.0, 3.0, 4.0, 5.0 mM
pH 8.3–0.5, 0.75, 1.0, 3.0, 4.0 mM

The time when the enzyme and NEM were mixed was marked as zero time of incubation. After appropriate periods of time, aliquots were withdrawn from the incubation mixture and diluted 50-fold into the reaction mixtures (50 mM urea, 1 mM EDTA, 20 mM phosphate buffer, pH 6.4, 7.4 and 8.3, respectively). The enzymatic reaction was started. After 5 min a sample of the reaction mixture was withdrawn and the amount of ammonia was determined. The concentration of ammonia was determined by the phenol-hypochlorite colorimetric method [13].

The amount of ammonia released in the reaction mixture for 5 min after addition of uninactivated urease was accounted as a control activity of 100%.

Thiols influence on inactivation of urease by NEM

The influence of monothiols (L-cys, GSH) and dithiol (DTT) on inactivation of urease by NEM was tested. The incubation mixture contained 0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 6.4, 7.4 and 8.3, respectively, 1 mM EDTA, 2.5 mM NEM and 1.25 mM thiol. The control sample instead of the thiol contained a proper volume of respective buffer. After 5, 10, 20 and 30 min of incubation, a sample of the incubation mixture was withdrawn and diluted 50-fold into the reaction mixture (50 mM urea, 1 mM EDTA, 20 mM phosphate buffer at respective pH). After 5 min the amount of ammonia was determined.

NEM-thiol-urease interaction test

The incubation mixtures contained 0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.4, 1 mM EDTA, NEM and the dithiol (DTT) or monothiol (L-cys).

The components of the incubation mixture were mixed according two procedures:

(a) urease was added to the mixture after a 20 min contact of NEM with the thiol. The final incubation mixtures contained:
   - 2.5 mM DTT and different concentrations of NEM: 0.375, 0.75, 1.25, 2.5 mM.
   - 2.5 mM NEM and different concentrations of thiol (DTT or L-cys): 0.375, 0.75, 1.25, 2.5 mM

(b) NEM was added to the mixture after a 20 min contact of urease with DTT. The final incubation mixtures contained 2.5 mM NEM and different concentration of DTT: 0.375, 0.75, 1.25, 2.5 mM.

The incubation mixture containing all components was incubated further for 20 min. Next, a sample of the incubation mixture was withdrawn and diluted 50-fold into the reaction mixture (50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA). The amount of ammonia released for 5 min was determined by the phenol-hypochlorite method.

Reactivation of NEM-inactivated urease

The reactivation of inactivated urease was studied in two ways, using DTT and by multidilution in the reaction mixture containing urea.

(1) The incubation mixture contained 0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 6.4, 7.4 and 8.3, respectively, 1 mM EDTA, 2.5 mM NEM.

After a 5 min incubation DTT was added. In a separate experiment DTT was added to incubation mixture, pH 6.4, after the total loss of urease activity (the incubation time over 300 min). DTT concentration in the incubation mixtures was equal to 2.5 mM. The activity of urease was determined before and after DTT addition.

(2) The samples of incubation mixture (0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 6.4, 7.4 and 8.3, respectively, 1 mM EDTA, 5 mM NEM) after incubation resulted in the total inactivation, were diluted 100-fold into reaction mixture: 50 mM urea, 20 mM phosphate buffer, pH 6.4, 7.4 and 8.3, respectively, 1 mM EDTA.

After appropriate periods of time, aliquots were withdrawn and the amount of ammonia was determined.

Theory and equations

An enzyme inactivator is a compound that produces irreversible inhibition of the enzyme because of formation of a stable covalent bond(s) between the inhibitor and essential functional groups of enzyme. Irreversibility, in that context, does not mean that the enzyme activity is never regained, but that the enzyme becomes dysfunctional for an extended, unspecified period of time. The inactivation is progressive with time reaching complete inhibition of the enzyme [14]. The anticipated reaction scheme for the inactivation of enzyme E by inactivator I is [15]:

$$E + I \underset{k_2}{\overset{k_1}{\Leftrightarrow}} EI \xrightarrow{k_3} EI^{\star} \tag{1}$$

where EI⋆ is a complex of inactivated enzyme with inactivator, k_1, k_2, k_3 are rate constants of the respective reactions. k_3 represents the inactivation rate constant at infinite inactivator concentration.

The total amount of enzyme equals:

$$E^o = E + EI + EI^{\star} = \varepsilon + EI^{\star} \tag{2}$$

The dissociation constant K_i for the reversible step of enzyme-inactivator interaction can be written as:

$$K_i = k_2/k_1 = I \cdot E/EI \tag{3}$$

The rate of formation of EI⋆ is expressed by:

$$-\frac{d\varepsilon}{dt} = k_3 EI \tag{4}$$

The solution of Equation (4) and application of Equation (3) has the following form:

$$\ln\frac{\varepsilon}{E^o} = -\frac{k_3}{1 + K_i/I}t \tag{5}$$

where ε/E^o defines the residual activity of the enzyme at time t and k_{app} is equal to:

$$k_{app} = \frac{k_3}{1 + K_i/I} \tag{6}$$

Residual activity as a function of incubation time represents an inactivation progress curve and can be experimentally obtained: $\varepsilon/E^o = f(t)$. In a model case, if the inactivator is added in large excess relative to enzyme concentration ($I \gg E^o$), the plot exhibits a pseudo-first-order kinetics:

$$\frac{\varepsilon}{E^o} = e^{-k_{app}t} \tag{7}$$

An approximation of exponential function to the experimental data allowed k_{app} to be obtained.

The relationship between k_{app} vs I in a double reciprocal system is linear:

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_i}{k_3}\frac{1}{I} \tag{8}$$

The data plotted in accordance with Equation (8) allowed determination of K_i and k_3 (k_3 denotes the inactivation rate constant). If $I \ll K_i$, $k_{app} = (k_3 / K_i)^{\star}I$ and k_3' can be set instead of k_3/K_i. It means that the kinetics is not distinguishable from a simple bimolecular mechanism: $E + I \rightarrow EI^{\star}$, and the data plotted in accordance with Equation (8) gives a straight line passing through the origin [15].

Results and discussion

Inactivation progress curves

The inactivation progress curves as a dependence of urease residual activity vs incubation time are presented in Figure 1. Urease was incubated with NEM at pH 6.4, 7.4 and 8.3, respectively. The used NEM concentrations were in the range 0.5–5.0 mM (details in Materials and Methods). NEM concentration in all incubation mixtures was in a large excess relatively to urease ($I \gg E^o$). It was shown that the increasing time of incubation resulted in the decrease of urease activity until the total loss of catalytic activity. The total inactivation of urease at pH 6.4 occurred after approximately 4 h (5.0 mM NEM, data not shown). Moreover, the increasing concentration of the inactivator caused the increase rate of inhibition. These results indicated that the inhibition of urease by NEM was time and concentration dependent. It was observed that the increasing pH resulted in the increasing inactivation rate. The process was slowest at pH 6.4 and most effective at pH 8.3. The systems pH 7.4 and 8.3 followed the monophasic pseudo-first-order kinetics of inactivation. The different mode was shown at pH 6.4. In that system the inactivation was much slower than at alkaline systems and showed

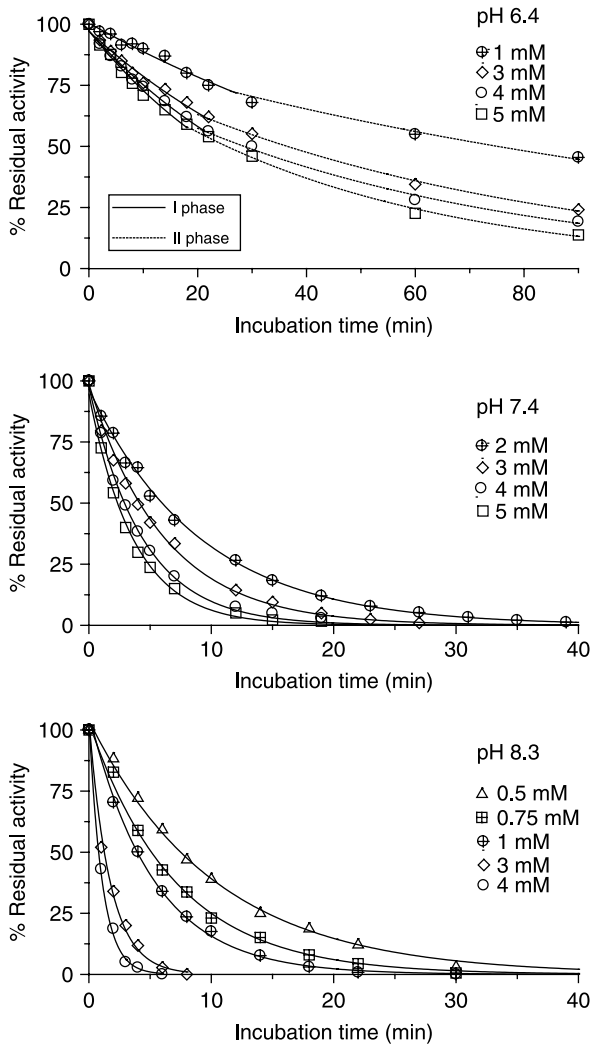

Figure 1. Inactivation progress curves as a dependence of urease residual activity *vs* incubation time at pHs 6.4, 7.4 and 8.3, for different NEM concentrations. Lines obtained as an approximation of the exponential function (Equation (7)) to the experimental data. At pH 6.4 each inactivation progress curve was fitted by two different exponential functions respectively to the phase of inactivation. NEM concentration is given numerically.

biphasic character. At the beginning phase the inactivation was fast further significantly slowed down. In order to calculate kinetic constants, the experimental data were approximated by exponential function (Equation (7)) and k_{app} were obtained. At pH 6.4 system each progress curve was fitted by two different exponential functions respectively to the phase of inactivation. The double reciprocal plots of k_{app} as a relation of NEM concentration are presented in Figure 2. The systems pH 7.4 and 8.3 produced straight lines passing through the origin. These results indicated that inactivation followed the simple bimolecular reaction and the inactivation rate was fast relative to formation urease-NEM complex. In that case only very weak reversible complex might be formed. The second order rate constants k_3' were obtained. At pH 6.4 system K_i and the first order rate constants k_3 were obtained for the fast and slow phase of the inactivation, respectively. The calculated kinetic constants are listed in Table I.

The second rate constant at pH 8.3 is significantly higher than at pH 7.4. The estimated inhibition rate constant at pH 6.4 for the fast phase is approximately 2-fold larger than for the slow phase. The calculated dissociation constant K_i of reversible complex formed in the fast and slow inactivation phases are of the same order.

Thiols influence on urease inactivation by NEM

The influence of thiols was tested at four time points of incubation. The results were compared with data of the thiol-free system. The used concentration of the inactivator was two times higher than concentration of the thiol. It was found that the coexistence of monothiol: L-cysteine or glutathione as well dithiol: dithiothreitol, with NEM in the incubation mixture slowed down urease inactivation (Figure 3). The correlation between the decrease of urease activity in the thiol-free system and system with the monothiol was observed. The general loss of urease activity in both systems was remained however it was slowed down in the presence of thiol. The inactivation was the least effective in the presence of DTT in all studied systems. At pH 6.4 and 7.4 the inactivation in the presence of DTT was almost not observed at studied range time. The significant difference in effectiveness between the monothiols and dithiol indicated on a different amount of the thiol provided -SH groups. Dithiol provided a double amount of –SH groups that resulted in a more effective reaction with NEM and finally in the urease inactivation decrease. The used monothiol concentration produced the equivalent of the thiol group concentration equal to half of applied

Table I. Kinetic constants of jack bean urease inactivation by NEM at 25 °C in 20 mM phosphate buffer, pH 6.4, 7.4, 8.3, respectively[a].

pH	Concentration range (mM)	$k_3/K_i = k_3$' (mM^{-1} min^{-1})	k_3 (min^{-1})	K_i (mM)
6.4	1.0–5.0	–	$k_3^{fast} = 0.070 \pm 0.008$ $k_3^{slow} = 0.048 \pm 0.005$	$K_i^{fast} = 7.0 \pm 0.8$ $K_i^{slow} = 7.5 \pm 0.9$
7.4	2.0–5.0	0.055 ± 0.006	–	large
8.3	0.5–4.0	0.19 ± 0.02	–	large

[a] The kinetic constants were determined from the fit of the data to Equation (8).

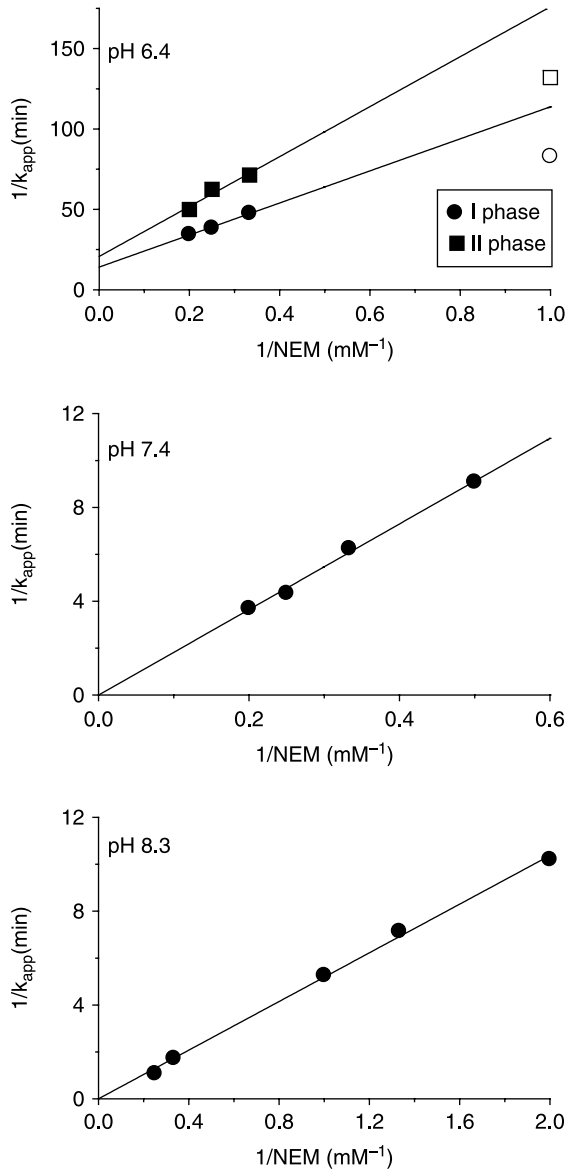

Figure 2. Dependence of k_{app} vs NEM concentration in a double reciprocal system, at pHs 6.4, 7.4 and 8.3. The data were fit to Equation (8). Points shown as empty symbols are not taken into account.

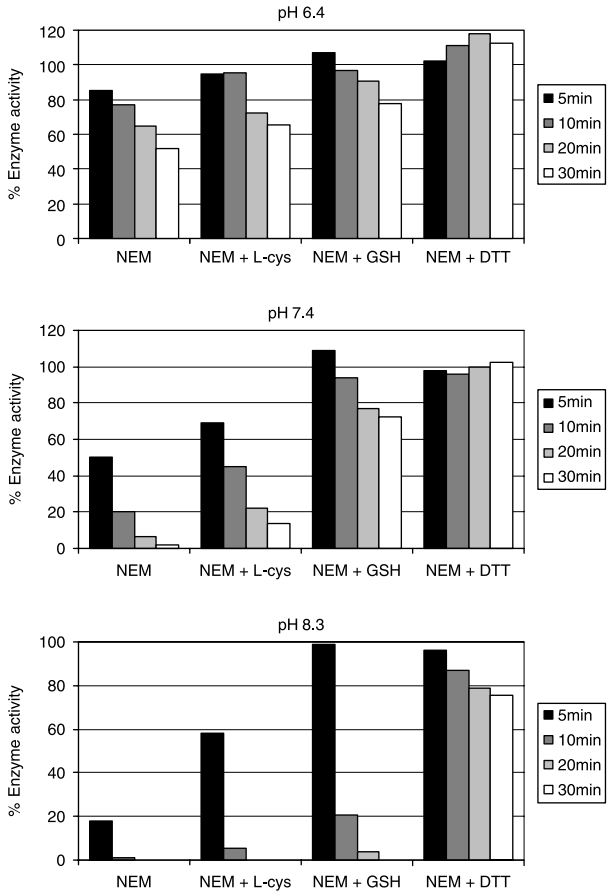

Figure 3. Thiol influence on urease inactivation by NEM relative to the control activity. The percent of the enzyme activity in the presence of NEM without the thiol is given for comparison. Concentration of the thiol: L-cysteine (L-cys), glutathione (GSH), dithiothreitol (DTT) was equal to 1.25 mM, respectively. NEM concentration was equal to 2.5 mM. Enzyme activity was determined after 5, 10, 20 and 30 min of incubation time, at pHs 6.4, 7.4 and 8.3.

NEM concentration (see "Inactivation progress curves" section). The reaction between NEM and monothiol just reduced the amount of inactivator. The inactivation process was slowed down but not stopped. Whereas dithiothreitol provided double amount of the thiol groups which eliminated NEM present in the system and the inactivation of urease was decayed. The direct NEM-thiol interaction confirmed NEM-thiol-urease interaction test. It was showed that urease remained highly active in spite of NEM presence in the incubation mixture if the thiol provided –SH group concentration was equal or higher than NEM concentration (Figure 4). On the other side, the amount of –SH groups lower than amount of NEM resulted in the high urease inactivation. Moreover the effect was independent of the order of components incubation. The incubation of urease with the thiol before NEM addition did not prevent the enzyme against NEM inactivation.

NEM is highly sulfhydryl reactive compound. The presence of the thiol and the thiol rich enzyme in the incubation mixture, offered the variety of possible interaction. The experiment showed that NEM-thiol interaction was strategic for the inactivation rate decrease.

Reactivation of NEM inactivated urease

The reactivation of NEM-inactivated urease was studied in two ways. In the first approach, DTT was applied. The method with DTT was thought to be promising due to high effectiveness in slowing down the NEM inactivation of urease. The obtained results did not confirm the above expectation (Figure 5). The applied ratio of NEM and DTT concentration was equal to 1:1 in all pH systems. DTT was added into pH 7.4 and 8.3 systems, in the course of inactivation

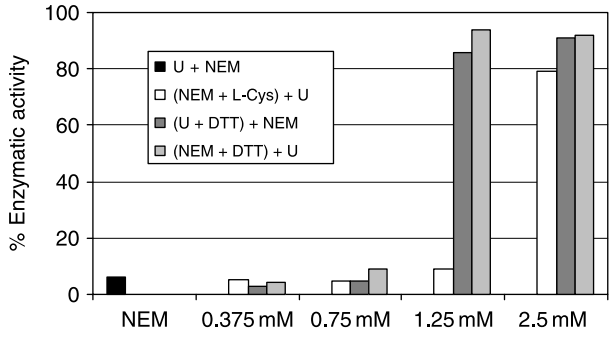

Figure 4. Influence of thiol concentration and order of components preincubation on urease inactivation by NEM. The initial 20 min preincubation mixture contained components given in brackets. The preincubation was continued further 20 min after addition of the last component (component given outside of brackets). The final preincubation mixtures contained 0.75 mg cm^{-3} urease (U), 20 mM phosphate buffer, pH 7.4, 1 mM EDTA, 2.5 mM NEM and dithiothreitol (DTT) or L-cysteine (L-cys). Enzyme activity was determined after 40 (20 + 20) min of preincubation time. The percent of the enzyme activity in the presence of NEM without the thiol is given for comparison. The thiol concentration is numerically given.

when urease activity was lowered up to ~40% of the control activity. After DTT addition the urease activity sustained the reached level without any increase or decrease. In pH 6.4 system DTT was applied at the beginning (the first phase; see Figure 1) and after the total urease inactivation. The reactivation effect in both approaches was very poor, suggesting the reactivation independence of time DTT addition. The experiment was repeated (at pH 7.4) for different NEM and DTT ratio: 2:1 and 1:2. The lack of the urease activity change

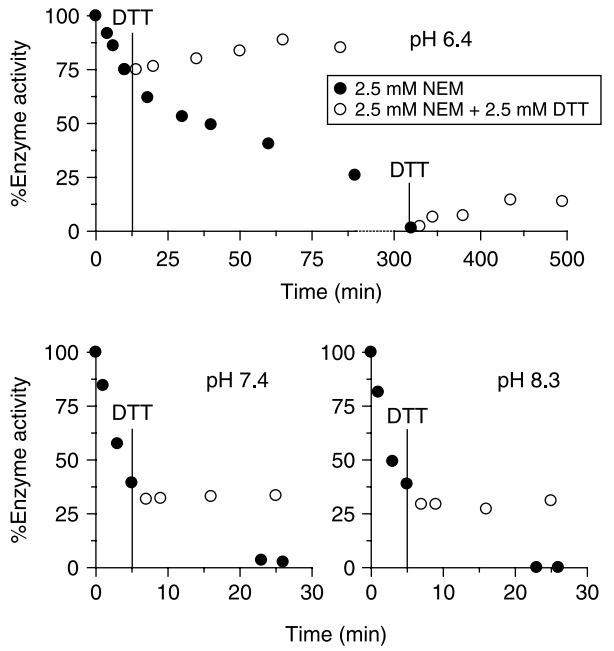

Figure 5. Reactivation of NEM inactivated urease by addition of dithiothreitol (DTT) at pHs 6.4, 7.4 and 8.3. Activity of urease inactivated by NEM (•) and after adding DTT (○). Additional details are provided in the text.

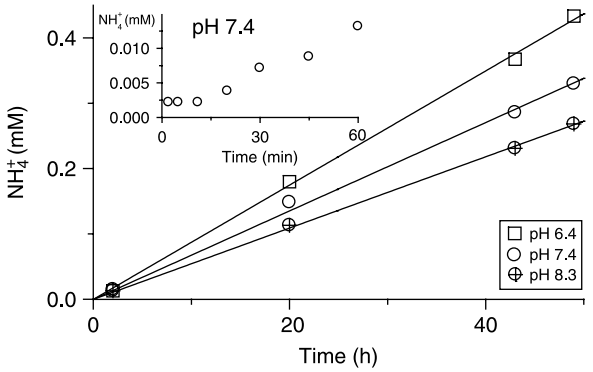

Figure 6. Reactivation of NEM-inactivated urease by 100-fold dilution in 50 mM urea at pHs 6.4, 7.4 and 8.3. Inset: beginning period of reactivation at pH 7.4 in a minute scale. The sample of preincubation mixture, after preincubation resulted in the total inactivation of urease by NEM, was diluted in the reaction mixture. After appropriate period of time, aliquots were withdrawn and the amount of ammonia was determined.

was also observed. Since DTT provided a double amount of thiol groups NEM could be totally bounded by the thiol in all assays. The result of that interaction was lack of further inactivation after DTT addition. Moreover, DTT did not affect urease-NEM complex (even in the system with a large DTT access) that caused negative reactivation effect. That evidence indicated that the urease-NEM complex was resistant for chemical modifications.

The second used method was multidilution of the inactive urease-NEM complex. NEM was preincubated with urease up to the total loss of activity. The incubation time was selected upon the enzyme activity measurement (Figure 1). The incubation mixture was diluted 100-fold into reaction mixture containing the substrate. The progress of the urease reactivation was monitored by the determination of realised ammonia. The obtained progress curves are presented in Figure 6. The linear increase of ammonia concentration pointed out the constant activity/amount of urease in the system and lack of the progressive enzyme release from urease-NEM complex. An insignificant amount of the active enzyme separated from complex occurred at the beginning just after dilution (Figure 6, inset) and there was no further active urease release. This proved the existence of strong covalent bounds in the urease-NEM complex.

Conclusions

The electrophile *N*-ethylmaleimide is used as a thiol reagent applied for determination of thiol groups in peptides and proteins. NEM is thought to be a thiol specific reagent, however, in spite of this, it also reacts with amino groups and imidazoles [16]. Urease contains numerous thiols with cysteine 592 important for catalytic activity. Moreover, active-site nickel ions are coordinated by histidine residues. These provide

opportunities for different possible ways for the NEM-urease interaction. However, numerous studies have pointed out that urease sulfhydryl groups were responsible for urease inactivation [11,12,17]. Our studies showed that the inactivation was more effective at pH 8.3 than pH 7.4 and 6.4. This accounted for the increased NEM affinity towards thiol with the pH increase. On the other side, the dissociated form of sulfhydryl residue is more reactive so the increased pH supports the inactivation. The reactivation experiment gave evidence of the high irreversibility of the urease-NEM complex. Moreover, this pointed out that the urease-NEM complex was resistant to chemical DTT modification so that the reactivation of the enzyme did not appear.

Independent of the applied pH, NEM totally inactivated urease; at pH 6.4 the process was biphasic and the slowest. Two stages of the inactivation could result from differentiation between the reactivity of urease thiols towards NEM at acidic pH.

Discussing pH influence on urease inactivation it seems that the spontaneous NEM hydrolysis could be neglected because of the slow rate compared to the NEM reaction with thiols. That assumption was confirmed by studies at pH 7.4 and 8.4. In alkaline environment the rate of inactivation was highest in spite of the fact that an alkaline pH favours NEM hydrolysis.

In conclusion, similar results from studies obtained at pH 6.4, 7.4 and 8.4 indicated that the mechanism of urease inactivation by NEM was pH-independent. However, the pH value significantly influenced the process rate.

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