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ARYLESTERASE IN BLOOD: IRREVERSIBLE INACTIVATION OF THE PLASMA ENZYME—II

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Abstract—Human plasma arylesterase is readily inactivated by urea or guanidine. This process is not reversed by dilution. Depending on the pH, Ca^{2+} and especially La^{3+} slowed down the denaturation considerably. Inhibitors which usually combine with SH-groups did not protect the enzyme. The interrelationship of the process with the ionization of various groups on the protein is discussed.

PREVIOUS publications indicated that the arylesterase of plasma or serum requires the presence of calcium for its activity.[1–3] Thus, it is inhibited by reagents which bind or procedures which remove calcium. Furthermore, it is extremely sensitive to rare earths;[1, 2] these ions inhibit the hydrolysis of phenyl acetate at 10^{-7} M, some even at a lower concentration. Arylesterase is possibly an SH-enzyme. Its activity is blocked by CMPS,* mercuric chloride, and heavy metals.[1, 2]

Since preliminary experiments showed that arylesterase is sensitive to urea,[2] the study of the inactivation of the enzyme seemed to offer more information about the effect of calcium and inhibitors.

MATERIALS AND METHODS

The source of arylesterase was Fraction IV-1 of human plasma.[2, 4] A stock solution, 10 mg/ml, was made up daily in sodium chloride solution (0·1 M) and stored on ice. If not otherwise indicated, final dilutions of the stock solution of 1 : 5 (2 mg of Fraction IV-1 per ml) were incubated at 0 °C with 3 M urea or 1·5 M guanidine hydrochloride in one of the following buffers: at pH 4·0, 4·8, 5·2, 5·7—0·1 or 0·08 M acetate; at pH 6·1, 6·7—0·02 M maleate; at pH 7·5 and 8·2—0·02 M Tris. When the effect of metal ions was studied, this mixture also contained the corresponding salt in the concentration indicated below. Immediately after mixing the enzyme with urea or guanidine, a sample was withdrawn, diluted finally to 1 : 100 v/v in tris buffer of pH 7·3 (0·025 M). After 5 min of pre-equilibration at 27 °C, it was assayed in a Cary recording spectrophotometer at $\lambda = 2800$ Å with a previously described technique.[2] The substrate was 1×10^{-3} M phenyl acetate. The rate of hydrolysis of the first sample was taken as "100 per cent, 0 time reading". Samples were withdrawn afterwards and assayed at regular intervals. The rates were expressed as fractions of the initial rate of hydrolysis.

* Abbreviations: CMPS = *p*-chloromercuriphenylsulfonic acid; Tris = tris-hydroxymethylaminomethane; EDTA and Ca-EDTA = sequestrene : disodium ethylenediaminetetra-acetic acid and its Ca complex; Chel DP = ethylenediamine di(o-hydroxyphenylacetic acid).

In some other studies, 1 mg of Fraction IV-1 per ml was incubated with 5 M urea, and a 1 : 20 v/v final dilution was used at pH 7·2 in the absorption cells of the spectrophotometer. Urea did not inhibit significantly at 0·25 M concentration.

RESULTS

Effect of urea

The inactivation of arylesterase by 3 M urea at pH 7·5 is shown in Fig. 1. Here the relative rate of hydrolysis of the substrate is plotted against time in the presence

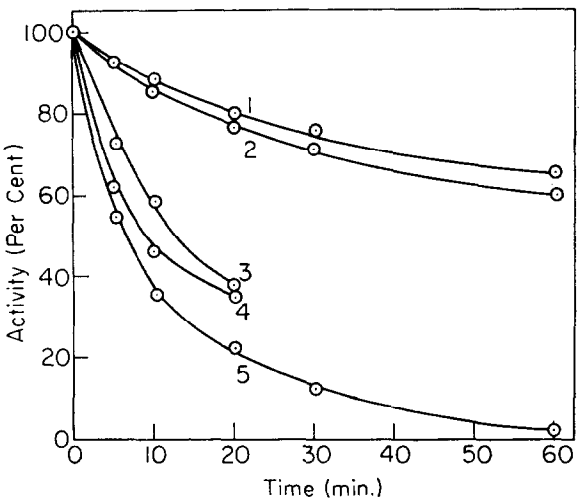

FIG. 1. Inactivation of arylesterase in 3 M urea.
(1) 1×10^{-3} M $LaCl_3$.
(2) 3×10^{-2} M $CaCl_2$.
(3) 1×10^{-2} M $MgCl_2$.
(4) 1×10^{-3} M $HgCl_2$.
(5) Control without added metal ions.

and absence of various agents. The addition of calcium chloride ($CaCl_2$), 3×10^{-2} M, or lanthanum chloride ($LaCl_3$), 1×10^{-3} M, slowed down this inactivation considerably. (The concentration of lanthanum chloride at the final dilution was 1×10^{-5} M, sufficient to have fully inhibited the arylesterase. To avoid this, the solution in the spectrophotometer contained 2×10^{-5} M Ca-EDTA to bind the La^{3+} present. Because La^{3+} has a much higher affinity (log $K = 15{\cdot}4$) for EDTA than does Ca^{2+} (log $K = 10{\cdot}6$), it easily replaces calcium in the complex.) Under these conditions, 3 M urea in 1 hr just about completely inactivated the enzyme, while from 60 to 65 per cent of the activity was preserved in the presence of calcium or lanthanum. When, instead of $LaCl_3$, another inhibitor, mercuric chloride ($HgCl_2$), 10^{-3} M, was used, only a fraction of the activity survived (see Fig. 1). (The 1×10^{-5} M final concentration of mercuric chloride also would have completely inhibited the enzyme; hence, we added glutathione, 2×10^{-4} M, as reactivator.[2] Cysteine could not replace glutathione in these experiments, because it is itself a good inhibitor of arylesterase: 85 per cent of the activity was blocked at 1×10^{-4} M concentration by both the D- and L-forms.[5]) A substance, CMPS, which reacts with SH-groups, was even less effective. Magnesium chloride ($MgCl_2$), 1×10^{-2} M, was as little active as mercuric chloride (see Fig. 1), but the activity of strontium chloride ($SrCl_2$), 1×10^{-2} M,

approached that of calcium chloride. The activity of calcium chloride was quite insensitive to variations in concentration from 3×10^{-3} M to 1×10^{-1} M.

The effect of lower and higher concentrations of urea was also explored. When 5 M urea was used, no activity was detectable in 5 min, but in the presence of 1×10^{-2} M calcium or 1×10^{-3} M lanthanum, 40 and 50 per cent, respectively, remained after incubation for 10 min.

In some experiments with 5 M urea, Ca-EDTA was replaced by another chelating agent, Chel DP. This compound has a very low affinity for calcium ions (log $K = 1{\cdot}6$). Although it binds lanthanum ions, it does not inhibit the enzyme,[2] unlike EDTA, which is a good inhibitor of the hydrolysis of phenyl acetate[1-3] or paraoxon[6] by arylesterase. When the arylesterase was incubated with $LaCl_3$, 1×10^{-3} M, in 5 M urea, and mixed after dilution with Chel DP, 2×10^{-4} M, no hydrolysis of the substrate was seen. But when, in addition to Chel DP, $CaCl_2$, 5×10^{-4} M, was added,

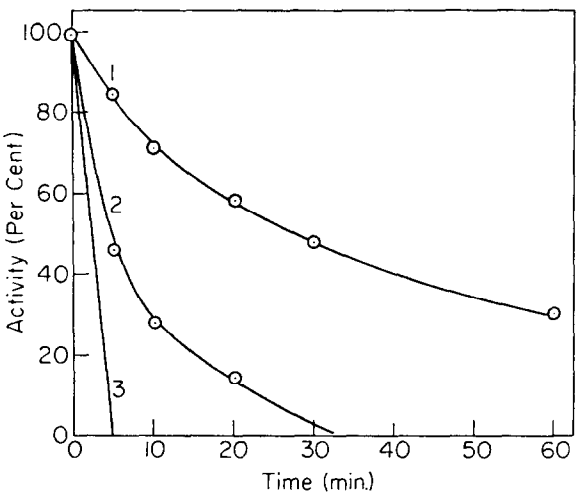

FIG. 2. Inactivation of arylesterase in 1·5 M guanidine.
(1) 1×10^{-3} M $LaCl_3$.
(2) 1×10^{-2} M $CaCl_2$.
(3) Control without added metal ions.

the rate was comparable to the one obtained with Ca-EDTA as chelating agent for $LaCl_3$. Apparently, in addition to the binding of lanthanum ions, the replacement of calcium ions is also necessary here for the reactivation. Lower concentrations of lanthanum chloride, 10^{-4} M, or 2×10^{-5} M, were only slightly less effective against 5 M urea. On the other hand, lowering the urea concentration to 2 M or 1 M greatly lowered the rate of inactivation of the unprotected enzyme. Controls with chelating agents alone gave negative results.

Effect of guanidine

Guanidine, as expected,[7] is a stronger inactivator of arylesterase than is urea. Experiments with guanidine, 1·5 M, and lanthanum chloride, 1×10^{-3} M, at pH 7·5, gave results resembling those with 5 M urea, but calcium protected against guanidine less than against urea (see Fig. 2).

In a control experiment the salt of another rare earth, gadolinium chloride, ($GdCl_3$), behaved much like $LaCl_3$. The effect of lanthanum chloride at lower concentrations of guanidine, 1 M, was apparently more prominent, when compared on the basis of curves like the ones shown in Fig. 2. However, this observation may not be significant, since the rate of inactivation in 1·5 M guanidine is too fast for accurate assay.

Guanidine was also a better inhibitor than urea. About half the activity of arylesterase was inhibited by a 5×10^{-2} M concentration.

Effect of ionic strength

At the pH of the experiments, guanidine is ionized[8] while urea is not. This results in a relatively low ionic strength ($\Gamma/2 = 0{\cdot}03$–$0{\cdot}1$) in the 3 M urea experiments and in a much higher one when guanidine hydrochloride is used. Therefore, the effect of moderate increase in the ionic strength was also investigated. The data revealed that sodium chloride, 0·6 M, did not significantly change the rate of denaturation by 3 M urea. However, the protection of the enzyme by $LaCl_3$, 10^{-3} M, or $CaCl_2$, 10^{-2} M, was more pronounced; only 20 or 26 per cent, respectively, was inactivated in 60 min at pH 7·5.

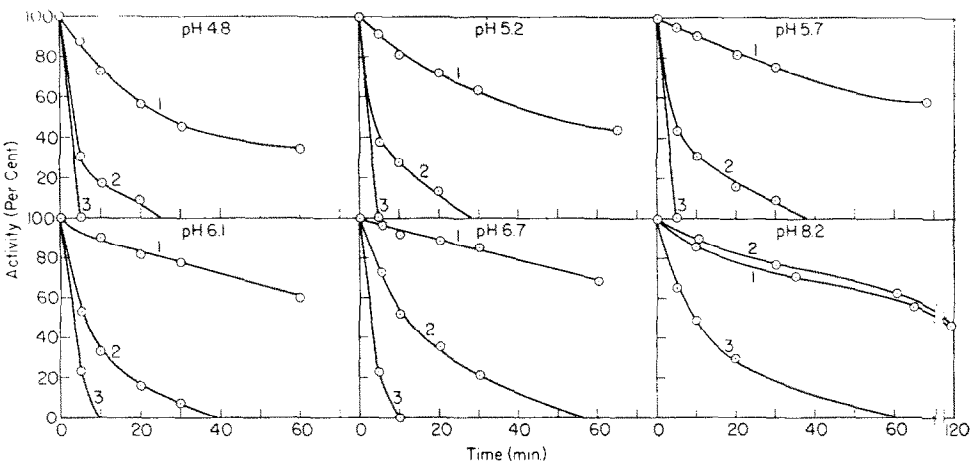

FIG. 3. Inactivation of arylesterase in 3 M urea at various pH values.
(1) 1×10^{-3} M $LaCl_3$.
(2) 1×10^{-2} M $CaCl_2$.
(3) Control without added metal ions.

Effect of pH

This and previous communications[1, 2] have shown that La^{3+} and Ca^{2+} exert a remarkably strong effect on the activity of arylesterase. In order to learn more about the sites of attachment of these ions we decided to study the inactivation of the enzyme at various hydrogen ion concentrations. To eliminate further variations in the experimental conditions, the activity after dilution was assayed at pH 7·3 ($\pm 0{\cdot}1$). Lanthanum, which at high concentrations readily forms insoluble hydroxides,[9] remained in solution at pH 8·2 when the solution contained urea.

In these experiments, the enzyme was incubated with urea in the presence and absence of added ions at pH values ranging from 4·0 to 8·2. At pH 4 the enzyme itself was not stable; more than half of the activity was lost in 40 min, but lanthanum slowed down this inactivation considerably. Also at this pH in 3 M urea or 1·5 M guanidine, the enzyme became inactive very rapidly—within 5 min. Lanthanum and calcium ions seemed to have a negligible effect. Possibly, under these circumstances, all the reactions were too fast to be measured with the present technique.

The experiments at pH values above 4 are shown in Fig. 3. Increasing the pH to 4·8 enhanced the effect of lanthanum while calcium stayed virtually inactive. A great rise in the activity of calcium was seen above pH 6·7. At pH 8·2 the two metals were about equal. The destruction of the unprotected enzyme was much slower at pH 8·2 than at 4·0. Fig. 4 summarizes the results. When the degree of inactivation in 3 M

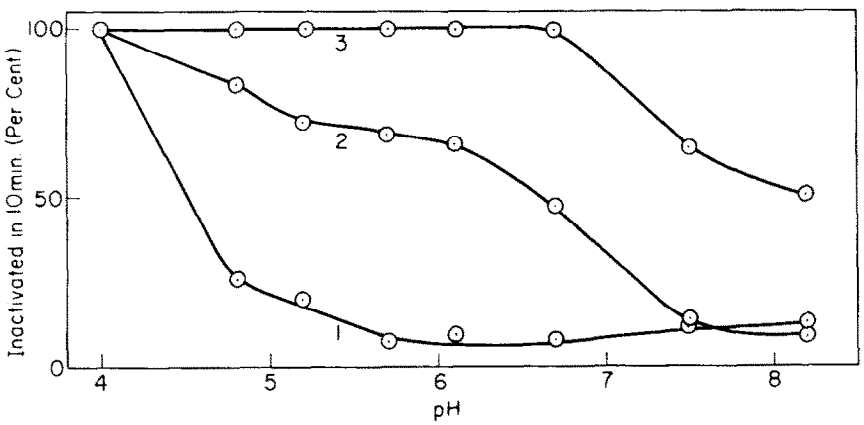

FIG. 4. Effect of pH on the inactivation of arylesterase by 3 M urea during the first 10 min.
(1) 1×10^{-3} M $LaCl_3$.
(2) 1×10^{-2} M $CaCl_2$.
(3) Control without added metal ions.

urea during the first 10 min is compared at various pH values, in the presence of $CaCl_2$, 1×10^{-2} M, or $LaCl_3$, 1×10^{-3} M, the differences between the two salts are easily observed. Thus while $LaCl_3$ became effective above pH 4, the activity of calcium was predominant only well above pH 6.

DISCUSSION

Arylesterase is an enzyme which occurs in the plasma of many members of vertebrata.[10, 11] These experiments showed that urea and guanidine readily destroy the activity of the human arylesterase at 0 °C. Depending on the pH, calcium, lanthanum, and also strontium, slowed down this process, which was not reversed by dilution. The effect of urea and guanidine on the proteins is usually interpreted as the destruction of the secondary structure through breaking of the hydrogen bonds.[12] However, urea is also a competitive inhibitor of a number of enzymes.[13] It has been indicated with some proteins that calcium can protect them against urea.[6, 14–17] On the other hand, acceleration of the process also has been observed.[18] The behavior of the enzymes

toward the denaturing agents varies individually with the pH.[7, 14, 16, 19–22] Since the purpose of these studies was to obtain some information about the groups on the surface of the enzyme which can combine with metal ions, the effect of urea was also studied at various hydrogen ion concentrations. Changes in the pH of the solution are usually accompanied by changes in the ionization on the protein. While studies with a partially purified protein can yield only a limited amount of information, the experiments which showed that the enzyme, after preincubation with either lanthanum chloride or mercuric chloride, needed a complexing agent for restoring the activity, indicated that these metals can combine with a group in the enzyme. The protection by $LaCl_3$ against urea may be attributable to that fact.

We are tempted to correlate the big change in the activity of lanthanum between pH 4·0 and 4·8 with the loss of a proton from a carboxy group on the protein. (Interference of the acetate in the buffer can be disregarded, because adding sodium acetate to the reaction at higher pH levels did not influence the rate. Other considerations make the possibility of interference by the maleic acid buffer unlikely.) The significant change in the effect of calcium chloride between pH 6·2 and 7·5 could be related to a change in the ionization of an imidazole group in the protein or to the change in the α-NH_2 groups of cystine.[23] Indeed, Chervenka[17] indicated that calcium might combine with an imidazole group in α-chymotrypsinogen. Imidazole is considered to be in the active center of a number of enzymes;[23] Mounter *et al.*[24] have made this suggestion for rabbit arylesterase.*

Agents which readily combine with the SH-groups on the protein, such as mercuric ions or CMPS, inhibited the enzyme but did not protect against urea.

It appears to us that the interrelationship of calcium with arylesterase has three facets. Calcium ions accelerate the activity of the enzyme. They are essential for the hydrolysis of the substrate and, finally, they stabilize the structure and protect it against denaturing agents. Lanthanum ions might combine with different groups than do calcium ions; however, they might inhibit the enzyme by replacing calcium ions, as indicated here and in previous experiments.[2]

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* In all of the above considerations the loss of protons by hydrated calcium or lanthanum and the enzyme–metal complexes was not evaluated.

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