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The Role of Sulfhydryl Groups in the Reactivation of Heat Inactivated Milk Alkaline Phosphatase

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A model system was developed for the study of the mechanism of reactivation of heat inactivated milk alkaline phosphatase. Sulfhydryl containing compounds were found to be effective but not compulsory components of the model system. The native and reactivated forms of the enzyme were not susceptible to N-ethylmaleimide (NEM) and *p*-chloromercuriphenylsulfonate (PCMS), whereas these reagents reacted with the heat inactivated enzyme so as to block the reactivation process. It appeared that a sulfhydryl group was activated by the heat treatment, but that this group disappeared during reactivation. This behaviour took place in a manner which was consistent with a mechanism other than reversible oxidation-reduction.

Upon certain conditions of storage, heat inactivated bovine milk alkaline phosphatase has been found to regain a portion of its original activity, and to give rise to an enzyme which cannot be distinguished from the native form. This process has been referred to as reactivation. A summary of the conditions related to this behaviour has been given by Lyster and Aschaffenburg[1]. The early theories of Wright and Tramer[2] do not include a consideration of the role of the sulfhydryl group, which appeared pertinent from the work of Hansson[3].

Therefore, this study was undertaken in an attempt to provide further insight into the mechanism of reactivation with special attention to the role of sulfhydryl. It seemed desirable to employ a model system for these studies, if possible, because the complexity of the whole milk system handicaps the interpretation of data. The model system used was developed independently and without knowledge of the simple system presented by Lyster and Aschaffenburg[1]. This might account for the differences between the composition of the two systems and the conclusions reached. However, many similar experiments were conducted and the results were in qualitative agreement.

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EXPERIMENTAL

Materials

Milk. The milk used in this study was graciously provided by the Central Laboratory, Mjölkcentralen, Stockholm. The raw milk was of normal commercial grade without attention to source or special handling.

Milk alkaline phosphatase. Purified milk alkaline phosphatase was prepared according to the procedure of Morton[4]. The enzyme routinely used corresponded to that obtained by Morton at stage 6 of his purification, and was of comparable specific activity. Following dialysis against distilled water the material was lyophilized and stored at 4°C over P_2O_5. The brown gummy material which remained in the flask after drying was discarded. Unsuccessful attempts were made to further purify the enzyme by chemical fractionation and ion exchange chromatography. Although fractions were obtained by the latter technique with specific activities about three times greater than Morton's most active preparation, the quantity of material was limited so as to prevent its use for detailed reactivation studies.

Calf intestinal alkaline phosphatase. Highly purified calf intestinal alkaline phosphatase was kindly supplied by Dr. L. Engström, Institute of Medical Chemistry, University of Uppsala. The material had an activity of 172 A_{287}/A_{280}, this unit being previously reported[5].

Substrate. The substrate, *p*-nitrophenylphosphate, was prepared as described by Bessey and Lowe[6].

Procedures

Assay. The assay consisted of incubating 4.0 ml of 0.1 M ethanolamine-HCl buffer, pH 9.8[7], 0.5 ml of 0.016 M *p*-nitrophenylphosphate, and 0.5 ml of the test material at 34 ± 0.1°C for 30 min. The reaction was stopped by the addition of 1.0 ml 25 % trichloroacetic acid (TCA) or saturated sodium tungstate (in cases when turbidity was not removed with TCA) and filtered if one of the components of the system gave rise to a precipitate. To 2.0 ml of the clear solution was added 1.0 ml of 1.9 M Na_2CO_3, and the liberated *p*-nitrophenol was determined in a Beckman DU Spectrophotometer at 400 mμ. The amount of liberated *p*-nitrophenol was evaluated by comparison with a standard curve. Assays were performed in order to be within the limits of Beer's Law and to maintain zero order kinetics. The unit of activity is defined as the μmoles of *p*-nitrophenol released per 30 min incubation.

Heat shocking. The enzyme solutions were routinely heat inactivated by a technique to be referred to as *shocking*. In this case 1.0 ml of the test material was placed into a pyrex test tube (6 × 60 mm) and tightly sealed with a rubber stopper. The tube was placed into an ice bath for at least 60 sec to allow chilling. Then it was transferred to an oil bath maintained at 125 ± 0.5°C, held for 60 sec without shaking, and rapidly returned to the ice bath.

Incubation. The phosphatase was reactivated regularly by placing 0.5 ml of the appropriate mixture into a rubber stoppered test tube and maintaining at 34°C for 30 or 60 min. A control sample consisting of 0.5 ml of the same mixture was assayed directly without the incubation step. In each case, the buffer and substrate were added directly to this tube for the assay. The holding period at 34°C, which permitted reactivation, will be referred to as the *incubation period*, and the process as *incubation*.

RESULTS

The model system. The basic components of the model system consisted of enzyme, a metal salt (normally $MgCl_2$), an organic compound, buffer, and a salt solution. The variables of pH and temperature were optimized, the time of holding at various stages of reactivation investigated, the concentration dependency established, and the order of addition of the various components considered. The amount of enzyme used was 0.06 ml of a 0.2 % solution, and the total volume before heating was 1.0 ml. Normally, 0.2 ml of an appropriate salt solution was added following shocking. After mixing by inversion, two 0.5 ml aliquots were removed for assay and incubation.

pH. The effects of variations of pH were studied with the following buffer systems over the indicated range: 1) 0.1 M NaH_2PO_4 – Na_2HPO_4, pH 5.0 – 7.0, 2) 0.1 M succinate-borax and 0.1 M succinate – NaOH, pH 5.0 – 6.0, 3) 0.1 M HOAc – NaOAc, pH 3.5 – 5.5, 4) 0.1 M HCl – KCl, pH 1.1 – 1.9, and 5) 0.1 M potassium biphtalate – HCl, pH 2.2 – 4.0. Maximum reactivation was obtained with the use of pH 3.5, 0.1 M HOAc-NaOAc buffer. It was learned that the enzyme could be maintained at this low pH for about one hour (either before or after heat treatment) before adding the salt solution without serious loss of reactivation.

Temperature effects. Various intervals of holding at 110, 125, and 140°C were investigated for heat shocking the samples, and reactivation was observed at all three temperatures. However, 60 sec at 125°C was found to be optimum and was used thereafter.

Reactivation in a system consisting of enzyme, cysteine, and magnesium was also observed if the enzyme was inactivated by holding at 63°C for 30 min instead of shocking. Repeated shocking of either milk or model systems prior to incubation resulted in progressive losses in reactivation.

The optimum temperature for incubation was found to be 35 ± 1°C, and 34°C was used throughout this study as recommended by McFarren[8]. Holding simple systems at higher temperatures ranging from 50–95°C for 60 sec after shocking and prior to incubation promoted decreases in reactivation with the greatest losses occurring at the highest temperatures.

Metal. Reactivation was enhanced if the enzyme was incubated in the presence of $MgCl_2$ or $Mg(OAc)_2$ as expected from earlier results[1,8]. It was found that an optimum concentration of magnesium was required and inhibition resulted if this concentration was exceeded. The magnesium was involved in the reactivation process since it was not effective if added after incubation. Also, the magnesium could be removed by dialysis after incubation, without serious loss of reactivation. The concentration of $MgCl_2$ finally employed in the 1.0 ml mixtures was 0.03 ml of a 4.93 M solution.

The role of zinc was investigated in view of recent evidence which suggested that this metal is part of alkaline phosphatase of different origin. The influence of $ZnCl_2$ upon reactivation was studied over a range of 1×10^{-2} M to 1×10^{-8} M. It was found that the amount of reactivation influenced by zinc was related to the presence of cysteine, magnesium, and inorganic phosphate. In the absence of magnesium, zinc could promote reactivation in the presence, but not absence of cysteine. Greater reactivation occurred if inorganic phosphate was included. Optimum reactivation took place with approximately 1×10^{-5} M $ZnCl_2$, 2×10^{-3} M inorganic phosphate, and 6.52×10^{-5} M cysteine. Higher concentrations of all components were inhibitory, and the magnitude of reactivation obtained with magnesium was never obtained. When an inhibitory level of zinc was used, the inhibition could be overcome by adding sufficient amounts of cysteine. Zinc was more effective in promoting reactivation when added to the mixture before rather than after the shock treatment.

Phosphate. The proper concentration of either inorganic sodium phosphate or β-glycerophosphate was found to enhance reactivation. The buffering capacity of these components must be considered when considering their effect, *e. g.* 0.02 M β-glycerophosphate promoted a shift in the pH from pH 3.55 to 4.20 before adding the NaOAc and from 5.80 to 5.90 after adding the salt. For these studies, the enzyme was dissolved in a buffer consisting of 0.01 M $Mg(OAc)_2$ adjusted to pH 7.5 with Tris. In the presence of magnesium, cysteine, and 0.1 M, pH 3.5 HOAc-NaOAc buffer optimum reactivation occurred with 0.002 M phosphate, higher concentrations being inhibitory. The phosphate was more effective when added before rather than after shocking. Optimum reactivation occurred with the same basic system with 0.002 M β-glycerophosphate, higher concentrations up to 0.02 M being inhibitory.

Organic component. In early studies it was learned that β-lactoglobulin at a concentration of 0.25 % would promote reactivation. This component could be replaced by an equivalent concentration of either α-casein or whole casein, but β-casein was only slightly effective. It was observed that an optimum concentration of each component was required. Also, in studies with α-casein in the presence of β-lactoglobulin, optimum concentrations of each component were required when the level of the other was held constant.

Since α-casein but not β-casein was partially effective in replacing β-lactoglobulin, it was decided to try sulfhydryl containing compounds. Bovine serum albumin (0.25 %), glutathione (2.3×10^{-4} M), and cysteine (6.52×10^{-4} M) could all serve as replacements for β-lactoglobulin.

Again, inhibition occurred with higher concentrations when these components were included in the model system. Thus, it appeared that the sulfhydryl group may be a necessary component, especially since N-ethylmaleimide (NEM) was able to inhibit the process. However, it was found that the oxidized glutathione (G-S-S-G), at a concentration of 3.25×10^{-4} M yielded reactivation, and alanine at a concentration of 0.98×10^{-2} M was effective. Ethylenediamine tetraacetic acid (EDTA) at a concentration ranging from 2.69×10^{-4} M to 10.7×10^{-4} M and ascorbic acid (6.52×10^{-4} M) were not able to replace the organic constituents.

Salts. When milk was used as a supplement to the model system it was boiled to destroy the native phosphatase. When studying acid treatment as a means of replacing boiling, it was

Table 1. Comparison of the ability of sodium acetate and sodium propionate to promote reactivation of milk alkaline phosphatase.

Sodium acetate		Sodium propionate	
pH	Units of activity	pH	Units of activity
5.6	0.007	5.8	0.006
5.7	0.026	5.9	0.037
5.75	0.059	6.0	0.048
5.8	0.043	6.1	0.101

learned that NaOAc was an effective activator. Therefore, 0.2 ml of a saturated aqueous solution of the salt at room temperature was added to the 1.0 ml mixture after the 125°C heat shock treatment, and before the incubation period. Saturated aqueous solutions of other salts were tried and $(NH_4)OAc$ and sodium propionate were effective whereas NaCl and $(NH_4)_2SO_4$ were not stimulatory, and sodium formate only slightly so. Sodium propionate was found to be more effective than NaOAc in preliminary experiments, and this effect was studied more closely by adjusting the stock solutions of NaOAc and sodium propionate to various pH values with NaOH to determine if this was a pH effect. These results are presented in Table 1. It is apparent that the difference in the effectiveness of these substances is not entirely related to pH. To further test this possibility, mixtures in the absence of NaOAc were adjusted to various pH values ranging from pH 4.55 to 8.6 with Na_2CO_3, but reactivation was not observed.

Inhibitors. Several components were found to inhibit reactivation when used in the reactivation mixtures. The sulfhydryl blocking agents N-ethylmaleimide (NEM) and *p*-chloromercuriphenylsulfonate (PCMS) were effective when added either before or after shocking. The anionic detergent, sodium dodecyl sulfate (SDS) and non-ionic detergent, Emasol 4130, were used in an attempt to replace the organic constituent, but were inhibitory by themselves and in the presence of β-lactoglobulin. The concentration of detergents ranged from 0.024 to 0.38 %. On one occasion, difficulty was encountered by using excess $CHCl_3$ as a preservative agent in the buffer solution, and it was later found that 2.0 % of $CHCl_3$ was sufficient to completely inhibit reactivation when added to the mixture before shock treatment.

It was found that the inhibitory action of NEM could be overcome by the presence of cysteine, however, cysteine was more effective in this case if added prior to rather than after heating. This suggested that NEM may bbe competing for sulfhydryl originating from the enzyme. This thought was supported when NEM was added to the model system consisting of alanine in lieu of cysteine and reactivation took place in the absence but not presence of NEM. The concentration of NEM used in the presence of alanine, magnesium, and β-glycerophosphate which completely inhibited reactivation was 9.22×10^{-5} M. However, lower concentrations of NEM ranging from $9.22 \times 10^{-6} - 9.22 \times 10^{-8}$ were activating. When the reactivation process was stopped at various intervals up to 60 min by either 1) the addition of NEM (final concentration of 9.22×10^{-5} M) and continued holding at 34°C or 2) by chilling duplicates without NEM to 0°C, parallel increments of reactivation were observed.

Order of addition. The effect of adding the various components of the model system before and after the heat shock treatment was investigated. The concentration of the additives before the addition of 0.2 ml NaOAc was 0.005 M phosphate, 6.52×10^{-4} M cysteine, and 0.143 M $MgCl_2$. A summary of the results obtained is presented in Table 2. If a given component was not included prior to heat shocking, it was added after cooling and prior to adding NaOAc. Apparently, inorganic phosphate was most essential and $MgCl_2$ the least essential component for optimum reactivation.

Also, a comparison was made of the effect of adding NaOAc and $MgCl_2$ before and after heat shocking with a system consisting of enzyme, water, and 50 % skimmilk. With this system, maximum reactivation occourred when the $MgCl_2$ was added prior to shocking and NaOAc after shocking.

Table 2. Relative effectiveness of the components of the model system when included before or after heat shocking.*

Order of decreasing reactivation	Components present before shocking		
	$MgCl_2$	Phosphate	Cysteine
1	+	+	+
2	—	+	+
3	—	+	—
4	+	+	—
5	—	—	+
6	+	—	+
7	+	—	—

*Components missing before heat shocking were added after shocking.

Holding periods. The effect of holding the samples at various stages of reactivation for various times was investigated. It was found that the samples could be maintained at pH 3.5 (either before or after heat shocking) for about one hour, whereas marked losses of reactivation occurred if they were held two hours. After addition of the NaOAc, the samples could be held overnight prior to reactivation with a negligible effect.

Reactivation was found to increase with the time of holding at 34°C after heat shocking up to approximately 10 hr with the milk system and 8 hr with a model system consisting of enzyme, magnesium, β-lactoglobulin, buffer, and NaOAc. Upon longer periods of holding, the amount of reactivation decreased, presumably due to inactivation of the reactivated enzyme.

Comparison of boiled and LTLT treated milk. When the organic component was replaced by either milk which had been placed in boiling water for 5 min or by milk which had been maintained at 63°C for 30 min, reactivation occurred. However, as expected from earlier studies[1], greater reactivation took place with the system containing the boiled milk. When cysteine was added to both systems, the results presented in Table 3 were obtained. In each case, double optima occurred: with tubes 1 and 4 with the boiled samples and tubes 3 and 6 with the 63°C treated samples.

Table 3. Influence of the concentration of cysteine in model system containing boiled and 63°C/30 min (LTLT) treated milk on reactivation.

Tube No.	Added cysteine conc. (M)	% Reactivation with added milk	
		63°C/30 min	Boiled
1	0	51	100
2	3.26×10^{-5}	83	88
3	6.52×10^{-5}	86	98
4	9.78×10^{-5}	74	100
5	1.30×10^{-4}	77	91
6	3.26×10^{-4}	83	91
7	6.52×10^{-4}	74	73
8	9.78×10^{-4}	53	53
9	1.30×10^{-3}	30	32

Reversibility of urea treatment in the presence and absence of NEM. A duplicate series of samples was treated reversibly in urea solutions ranging from zero to 6.4 M urea, in the presence of NEM. The samples were maintained in an ice bath in the presence of urea for 5 min, and then diluted 1:25 with buffer and substrate for assay. No difference between the level of reactivation was noted with these samples, contrary to what might be anticipated if sulfhydryl was liberated by the urea treatment. Only 80 % of the original activity was recovered in the case of the sample treated with 6.4 M urea, whereas only a slight loss of activity was observed with 4.8 M and lower concentrations of urea.

Calf intestinal alkaline phosphatase. Attempts were made to substitute highly purified calf intestinal alkaline phosphatase for milk alkaline phosphatase in the model system, but reactivation did not occur. Nearly five times more intestinal phosphatase was used than the amount of milk phosphatase normally employed (in terms of *p*-nitrophenol liberated per unit time). To insure that the milk phosphatase dit not contain an unidentified activator, both enzymes were treated together, but reactivation occurred to the same extent as when the milk enzyme was used alone.

In a related experiment, the highly purified milk alkaline phosphatase which was obtained from DEAE chromatography, was used in the model system, and reactivation was observed to the same extent as with the partially purified material normally employed.

DISCUSSION

Detailed discussion of the relationship between reactivation and treatment of various milk fractions, will be presented in another publication[9]. The purpose of this presentation was to show how the results obtained with the model system may be related to the behavior of the complex milk system with special reference to the role of sulfhydryl.

From the results of this investigation and of others[1], the lack of specificity of the components of the model system is supported. The role of metals in the mechanism of action of the enzyme has not been elucidated and it is not possible to define their function in the model system. Although zinc has been related to the active site of phosphatase[10], magnesium was a more effective component of the model system. Inorganic phosphate has been found to incorporate into intestinal alkaline phosphatases[11], and its protective action in reactivation could be similar to that of the substrate, β-glycerophosphate. No buffer ion specificity directly related to the reactivation process was observed. It would appear that a specific chelating agent is required for reactivation with the systems employed. Heavy metal contamination could be involved, since it would be expected that enzyme derived sulfhydryl would be susceptible to heavy metals. Apparently, EDTA competes for a metal which is essential for activity, presumably zinc. Although sulfhydryl is an effective agent promoting reactivation, its replacement by alanine indicates that there is not a specific sulfhydryl requirement. The fact that both GSH and G-S-S-G permitted reactivation suggests that the O/R potential of the system is not important for reactivation. Ascorbic acid did not allow reactivation when present in the absence of cysteine, however, reactivation took place if cysteine was included. Also, the low pH of 5.8 used for incubation is not favorable for the oxidation of sulfhydryl. In view of these observations, the release and disappearance of sulfhydryl does not seem to take place by reversible oxidation-reduction in a manner characteristic of other proteins, *e. g.*[12]. The fact that the substrate, β-glycerophosphate, does not protect the heat-inactivated form of the enzyme in the presence of NEM suggests that the liberated sulfhydryl group is removed from the active site. The function of the salt added after heat shocking is not known, and one can only speculate. Apparently, there is some specificity

involved which is related to a function other than pH or ionic-strength and it is tempting to suggest that the salt influences hydrophobic bonding between the enzyme and solvent. The reactivation mechanism may be summarized by the equation:

$$\text{Native enzyme} \xrightarrow[\text{Heat Shock}]{Mg^{2+},\ \text{alanine, pH 3.5, 0.1 M NaOAc–HOAc}} \text{Inactive enzyme-SH} \xrightarrow[34°C/30\ \text{min}]{\text{NaOAc}} \text{Active enzyme}$$

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