MECHANISM OF THE REVERSIBLE INACTIVATION OF TWO METAL-FLAVOPROTEINS BY CHELATORS AND THE FUNCTION OF THEIR METAL COMPONENTS*

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Received June 19, 1961

The experimental basis of the once widely-held hypothesis that the main function of the metal components of metal-flavoproteins is to catalyze oxido-reduction between the flavin group and one-electron acceptors is extremely meager (Singer and Massey, 1957). In fact, with the exception of nitrate reductase (Nicholas and Nason, 1954), proof that metals participate in the catalytic action of metal-flavoproteins is still lacking. The main reason for this circumstance is that reversible removal of the metal, accompanied by reversible inactivation, usually cannot be demonstrated owing to the strength of the flavoprotein-metal bond and/or the instability of the enzymes under the conditions of resolution.

It was recently found that two new metal-flavoproteins, the D-α-hydrox acid dehydrogenase (DHAD) (Curdel et al., 1959; Boeri et al., 1960) and the D-lactic cytochrome reductase of yeast (Gregolin et al., 1961; Gregolin and Singer, 1961a) are readily inactivated by metal chelators in a reversible manner. The chief interest of these observations was that if the reversible inactivation represented a genuine resolution of the holoenzymes, then the two D-lactic enzymes would provide valuable experimental tools for the unambiguous study of the function of metals in at least this group of flavoproteins. Curdel and Labeyrie (1961) and Stachiewicz et al. (1961) in fact, stated that the inactivation of DHAD by ethylene diamine tetracetate (EDTA) is a genuine resolution of the holoenzyme and could not be due

* Supported by grants from the National Heart Institute, U. S. Public Health Service (H-1995), the American Heart Association, and by contract No. Nonr 1656 (00) between the Office of Naval Research and this Institute.

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to the formation of an inactive holoenzyme-EDTA chelator. On the other hand, Tubbs (1960) favors the view that the inactivation of DHAD by chelating agents is due to the binding of chelator to the metal while leaving the metal attached to the enzyme. At least three mechanisms may play a role in the inactivation of these enzymes by chelators:

(1) Fp-metal + chelator → Fp-metal-chelate (inactive)

(2) Fp-metal + chelator → Fp (inactive) + metal-chelate

(3) Fp (inactive) → Denatured Fp (inactive, not reactivable).

It is important to distinguish between these mechanisms, since if the first one is operative, reactivation by added metals following the removal of non-protein-bound chelator (by dialysis or gel exclusion) merely reflects the ability of various metals to react with protein-bound chelator, while if the second one is operative, the efficiency of reactivation by different metals depends on their ability to combine with the flavoprotein (Fp) and yield an active holoenzyme. The third mechanism would suggest that the metal may also stabilize the structure of the enzyme.

When a purified preparation of DHAD is treated for 10 minutes at 30° with 0.8 mM EDTA under conditions similar to those employed by Curdel and Labeyrie (1961), the enzyme is about 90% inactivated. Following passage through Sephadex G-50 (equilibrated with 0.01 M phosphate, pH 7.5) to remove non-protein-bound EDTA, incubation at 30° under N_2 without added metals leads to extensive reactivation; at 0° only a slight spontaneous reactivation is observed (Fig. 1). Throughout this period the activity of a sample similarly treated except for the omission of EDTA remains constant. Similar results were observed when the inactivation was performed by dialysis against EDTA, followed by a second dialysis against phosphate or when o-phenanthroline was substituted for EDTA.

Fig. 2 illustrates the spontaneous reactivation of a highly purified preparation of D-lactic cytochrome reductase (Gregolin and Singer, 1961b), following inactivation by o-phenanthroline. The rate of reactivation as well as the equilibrium reached depend greatly on the temperature. When the temperature is changed after equilibrium is attained, a new equilibrium is established corresponding to the new temperature. The maximum reactivation reached (about 40% under these conditions) may represent that part of the enzyme which was in the form Fp-Me-chelate. These experiments offer proof of the occurrence of reaction (1).

Evidence for the occurrence of reaction (2) is indirect and is based on two observations. First, reactivation of the reductase by added Zn^{++}, following inactivation by dialysis against a suitable metal chelator,

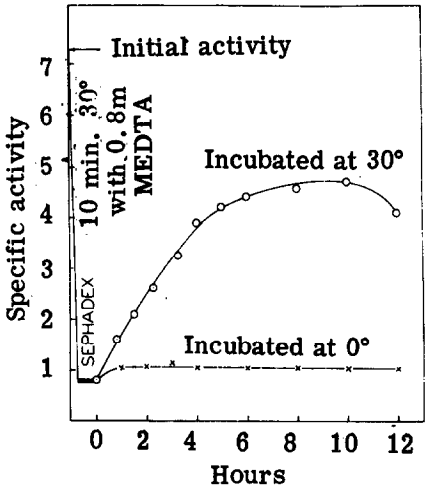

Fig. 1. Spontaneous reactivation of DHAD at pH 7.5, under N_2, following inactivation by 0.8 mM EDTA and passage through Sephadex G-50. Aliquots were assayed at the times indicated as per Boeri et al. (1960).

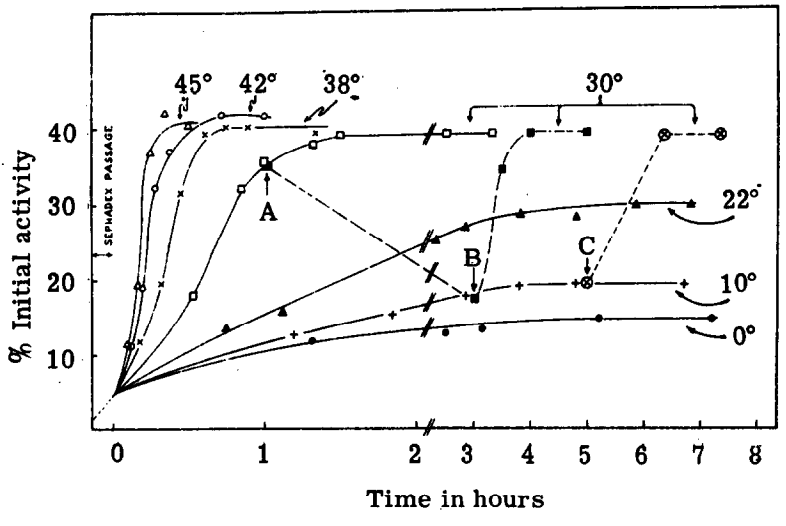

Fig. 2. Spontaneous reactivation of highly purified D-lactic cytochrome reductase in 10 mM phosphate, pH 6.5, under N_2, at the temperatures indicated, following inactivation by anaerobic dialysis overnight against 3.5 mM o-phenanthroline in 10 mM phosphate, pH 6.5, and passage through Sephadex G-25. Aliquots were assayed as per Gregolin and Singer (1961a). The temperature was changed at points marked A, B, and C. With this enzyme mere passage through Sephadex yields partial reactivation (reversal of reaction (1)).

is greater than the extent of spontaneous reactivation. Second, following inactivation by dialysis against chelators and reactivation of DHAD by added metals, the relative rates of oxidation of various substrates (V_{max}) and the corresponding apparent K_M values depend on the metal employed for reactivation, and the values obtained differ from the constants of the untreated enzyme. Similarly, following inactivation by o-phenanthroline, the apparent K_M of the Zn^{++}-reactivated reductase for D-lactate is different from that of the original enzyme. This behavior would not be expected if the added metal functioned merely by removing the chelator (reversal of reaction (1)). Reversible resolution with regard to both FAD and metal has recently been accomplished by acid $(NH_4)_2SO_4$ treatment (Gregolin and Singer, 1961b).

The reason why reaction (3) is believed to play a role in the inactivation by chelators is that the reactivability of either enzyme by added metals decreases with time if the sample is incubated for prolonged periods following removal of the unbound chelator (cf. also Curdel and Labeyrie, 1961). Since under identical conditions the untreated enzymes retain their activity, it would appear that these apoenzymes may undergo denaturation more readily than the holoenzymes.

It appears quite probable, then, that the inactivation of these enzymes by metal chelators does not proceed by a single mechanism. The extent of contribution of reactions 1, 2, and 3 to the total inactivation is probably a function of the experimental conditions. This may explain the divergent observations of Curdel *et al.* (1959) and Boeri *et al.* (1960). The former authors have found only Zn^{++} and, to a lesser extent, Co^{++} effective in reversing the inactivation of DHAD by EDTA, while Boeri *et al.* (1960) and Gregolin *et al.* (1961) have reported reactivation by various divalent metals.

The variations in the relative rates of oxidation of substrates and of their apparent K_M values depending on the metal employed for reactivation, referred to above, were the original basis of the hypothesis that the primary function of the metal in these two enzymes is to bind the substrate (Gregolin *et al.*, (1961; Gregolin and Singer, 1961a). In accord with this idea the apparent K_M for the reactivating metal is also the function of the substrate present. The fact that the metal is necessary for the initial reduction of the flavin has now been directly demonstrated for DHAD; the EDTA-treated, dialyzed enzyme is not reduced at 450 mμ by D-lactate, while on addition of Zn^{++} rapid bleaching occurs. The same function of the metal has been proposed, independently, by Tubbs (1960) for the DHAD of kidney.

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