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IRON: A POSSIBLE HETEROTROPIC EFFECTOR OF PROLYL HYDROXYLASE

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Summary

The mechanism of ferrous ion binding to prolyl hydroxylase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) was studied according to Koshland's curve-fitting procedure (Cornish-Bowden, A. and Koshland, Jr., D.E. (1970) Biochemistry 9, 3325—3336). The calculated data obtained by means of the unrestricted Adair equation were found to provide an adequate fit with experimentally obtained values, whereas those obtained on the basis of Michaelis-Menten kinetics did not. This suggests that prolyl hydroxylase could be an allosteric enzyme under positive heterotropic control.

There have been no reports in the literature that prolyl hydroxylase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, EC 1.14.11.2) is a regulatory enzyme. However, in view of its tetrameric structure [2,3] and its reaction mechanisms requiring several cofactors [4,5], there is a strong possibility that it may be an allosteric enzyme. The best method of testing this hypothesis is by detailed analysis of the Fe^{2+} saturation curve for prolyl hydroxylase by Koshland's methods [1].

The classical saturation curve for binding of a ligand X to a multisubunit protein E can be expressed in the form of Eqn. 1:

$$N_X = \frac{K_1[X] + 2K_1K_2[X]^2 + 3K_1K_2K_3[X]^3 + 4K_1K_2K_3K_4[X]^4}{1 + K_1[X] + K_1K_2[X]^2 + K_1K_2K_3[X]^3 + K_1K_2K_3K_4[X]^4} \quad (1)$$

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where N_X = number of mol of X per mol of protein.

In this equation, the constants K_1, K_2, K_3, and K_4 represent the binding of the first, second, third, and fourth molecules of ligand as shown in Eqn. 2:

$$K_1 = \frac{[EX]}{[E]\,[X]}\ ,\quad K_2 = \frac{[EX_2]}{[EX]\,[X]}\ ,\quad K_3 = \frac{[EX_3]}{[EX_2]\,[X]}\ ,\quad K_4 = \frac{[EX_4]}{[EX_3]\,[X]} \tag{2}$$

In general, for equations which are non-linear the standard statistical method of obtaining a least-squares fit are not applicable. Also, biological models may be more involved than those of inorganic complex ions. However, Koshland and his co-workers [1] developed a procedure which allows quantitative fitting of saturation curves and the determination of standard errors of the assigned parameters. In their method, the curve-fitting procedure could be more readily applied if the standard binding equation were written in the form of the unrestricted Adair equation, Eqn. 3:

$$N_X = \frac{\psi_1[X] + 2\psi_2[X]^2 + 3\psi_3[X]^3 + 4\psi_4[X]^4}{1 + \psi_1[X] + \psi_2[X]^2 + \psi_3[X]^3 + \psi_4[X]^4} \tag{3}$$

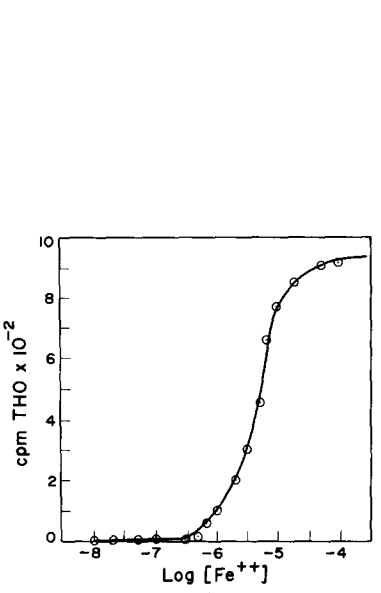
The evaluation of the ψ values then can lead directly to the calculation of the individual binding constants as shown in Eqn. 4:

$$\psi_1 = K_1,\quad \frac{\psi_2}{\psi_1} = K_2,\quad \frac{\psi_3}{\psi_2} = K_3,\quad \frac{\psi_4}{\psi_3} = K_4. \tag{4}$$

Starting values or 'guesses' of the individual ψ values were used as input information for the computer program in order to start the curve-fitting procedure.

The computer generates the final ψ values by the procedure described by Koshland and then calculates the individual binding constants, i.e., K_1, K_2, K_3, and K_4. A statistical factor $i/(n-i+1)$ is applied to these constants to obtain new constants called intrinsic binding constants (K_i'). By comparing the relationship of the calculated intrinsic binding constants to those given by Koshland for various theoretical models for a tetrameric protein, a prediction may be made of the type of model that seems to best describe the enzyme-ligand binding. Scatchard and Hill plots of the experimental data compared to the theoretical prediction are useful in confirming the model.

Prolyl hydroxylase was isolated and purified from the skins of newborn rats by the procedure of Rhoads and Udenfriend [6,7]. Enzyme activity was measured using tritiated prolyl protocollagen as substrate [7,8]. Prolyl hydroxylase activity was studied at various Fe^{2+} ion concentrations in the complete reaction mixture and plotted as a function of log (Fe^{2+}) (Fig. 1). The curve obtained was extrapolated to obtain maximum activity. At this activity, 100% saturation was assumed. The % saturation for all other Fe^{2+} ion concentrations was determined by comparing the activity at each concentration to the maximum activity. These data (Fig. 2) are based on the



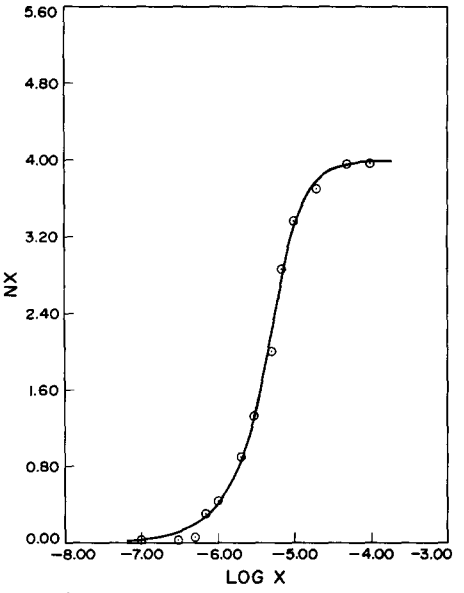


Fig. 1. Effect of [Fe^{2+}] on prolyl hydroxylase activity. [Fe^{2+}] in mol/l. Activity indicated by released tritiated water (THO) in counts/min (cpm).

Fig. 2. Saturation function for Fe^{2+} ions as determined experimentally and fitted to theoretical curve based on unrestricted Adair equation. X = [Fe^{2+}] in mol/l. NX = mol of Fe^{2+}/mol of enzyme. Experimental data (○). Theoretical curve (—).

assumption that 4 mol of Fe^{2+} are bound per mol of enzyme at maximum activity.

Fig. 2 illustrates the saturation curve generated from the data obtained from Fig. 1. The theoretical saturation curve (solid line, Fig. 2) was obtained by use of the unrestricted Adair equation. The shape of this saturation curve also resembles that of the original enzyme activity vs. log [Fe^{2+}] curve [Fig. 1]. Furthermore, the experimental points in Fig. 2 provide a good fit with the theoretical curve.

The intrinsic binding constants were calculated and found to be: $K'_1 = 9.0 \times 10^4$, $K'_2 = 1.9 \times 10^5$, $K'_3 = 6.8 \times 10^4$, $K'_4 = 3.1 \times 10^6$. From the pattern of these constants, i.e., $K'_1 < K'_2 > K'_3 < K'_4$, simple positive co-operativity is indicated overall [9]. This assumption is confirmed by the shapes of the Scatchard and Hill plots illustrated by Figs. 3 and 4 respectively.

However, when the same curve-fitting procedure was applied to the identical data using the Michaelis-Menten Model ($K'_1 = K'_2 = K'_3 = K'_4$), it is apparent that none of the curves illustrated by Fig. 5—7 fit the data. Therefore, it is concluded that the effect of Fe^{2+} ions on prolyl hydroxylase activity does not follow simple Michaelis-Menten kinetics. A symmetry model in which $K'_1 = K'_2 = K'_3 < K'_4$ might be postulated although the Scatchard plot (Fig. 3) contains no portion easily fitted by a straight line.

Alternatively, a sequential model could be proposed in which the binding of the first Fe^{2+} ion makes it easier for the next Fe^{2+} ion to bind which in turn influences subsequent binding. From the pattern of the intrinsic binding constants, it is possible that this model has both positive and negative co-op-

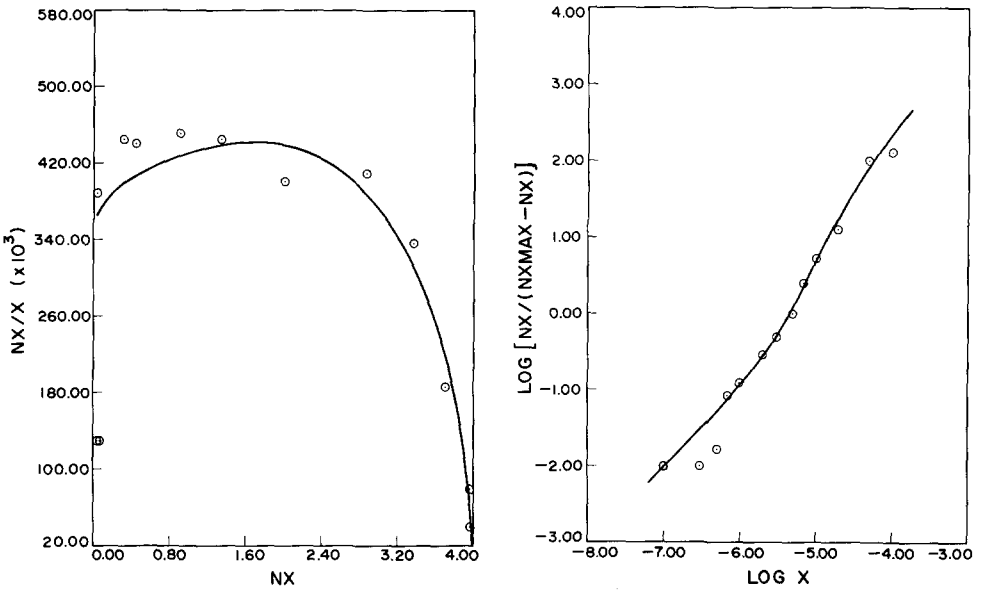


Fig. 3. Scatchard plot of experimental data used to obtain saturation curve shown in Fig. 2. X = $[Fe^{2+}]$ in mol/l. NX = mol of Fe^{2+}/mol of enzyme. Experimental data (○). Theoretical curve (—).

Fig. 4. Hill plot of experimental data used to obtain saturation curve shown in Fig. 2. X = $[Fe^{2+}]$ in mol/l. NX = mol of Fe^{2+}/mol of enzyme. Experimental data (○). Theoretical curve (—).

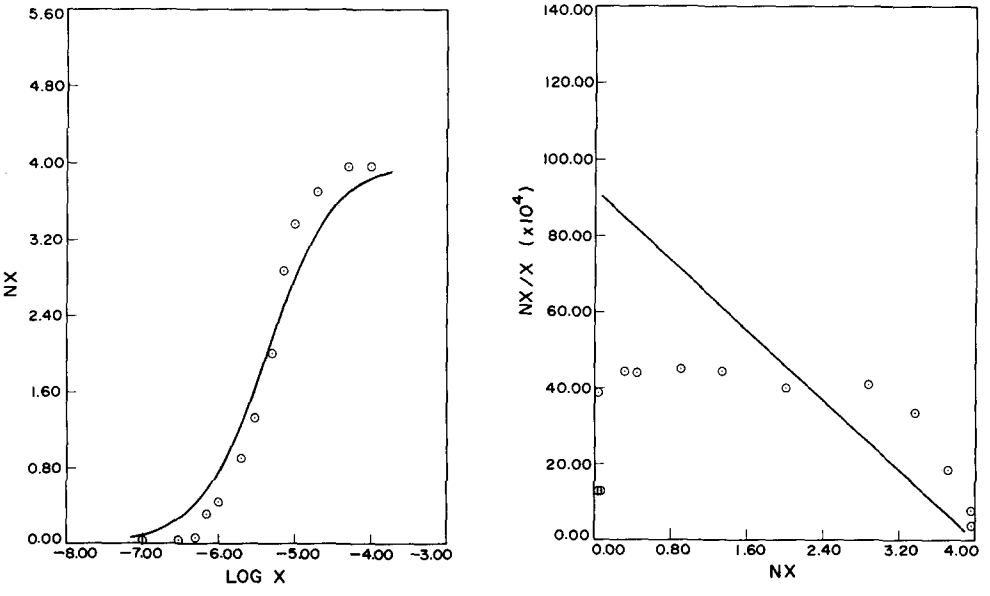


Fig. 5. Saturation function for Fe^{2+} ions as determined experimentally and fitted to theoretical curve based on Michaelis-Menten equation. X = $[Fe^{2+}]$ in mol/l. NX = mol of Fe^{2+}/mol of enzyme. Experimental data (○). Theoretical curve (—).

Fig. 6. Scarchard plot of experimental data used to obtain curve shown in Fig. 5. X = $[Fe^{2+}]$ in mol/l. NX = mol of Fe^{2+}/mol of enzyme. Experimental data (○). Theoretical curve (—).

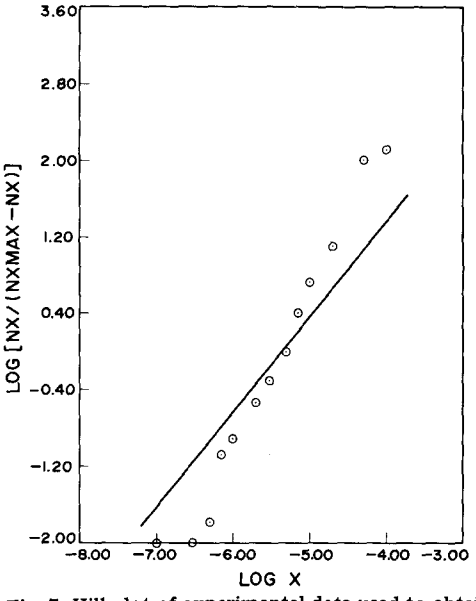


Fig. 7. Hill plot of experimental data used to obtain curve shown in Fig. 5. X = $[Fe^{2+}]$ in mol/l. NX = mol of Fe^{2+}/mol of enzyme. Experimental data (○). Theoretical curve (—).

erativity. This is similar to the case of yeast glyceraldehyde-3-phosphate dehydrogenase described by Cook and Koshland [10]. Positive co-operativity would make prolyl hydroxylase more responsive to changes in environmental Fe^{2+} ion concentration. Negative co-operativity, on the other hand, would make the enzyme less sensitive to such changes.

The present analysis does not allow discrimination between these possibilities. It is clear, however, that the binding of Fe^{2+} to prolyl hydroxylase follows non-Michaelis-Menten kinetics and that the heterotropic nature of the enzyme has been demonstrated.

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