Avidin Is a Slow-Binding Inhibitor of Pyruvate Carboxylase†

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ABSTRACT: In the presence of avidin, progress curves for the pyruvate carboxylase (EC 6.4.1.1) reaction show a marked decrease in the rate of reaction over a period of several minutes. These curves are quantitatively described by an equation in which the rate of catalysis by the enzyme undergoes a first-order decay from a high initial velocity to a final velocity which is close to zero. The initial velocity of the reaction is independent of avidin concentration while the decay constant increases linearly with increasing concentrations of avidin. These findings are in agreement with a model in which there is slow equilibration between free enzyme and the enzyme–avidin complex. Alternative models involving slow isomerization of the free enzyme or of an enzyme–avidin complex are not consistent with the data. From the slope of the decay constant vs. avidin concentration plot, the rate constant for formation of the enzyme–avidin complex was calculated to be (1.42 ± 0.09) × 10^5 M^{-1} s^{-1} for the enzyme isolated from chicken liver. The intercept of this plot, which is equal to the rate constant for dissociation of the enzyme–avidin complex, was not well-defined by the data but was estimated to be less than 2 × 10^{-3} s^{-1}. In view of this uncertainty, an alternative procedure was used to obtain a more reliable value. This procedure involved following the regeneration of enzymic activity upon incubation of the enzyme–avidin complex with biotin and yielded a value of (1.45 ± 0.13) × 10^{-5} s^{-1} for the dissociation rate constant. The enzyme–avidin complex undergoes a reaction which renders the inhibition of the enzyme irreversible and which occurs at a rate that is 5 times greater than the rate of dissociation of the avidin from the enzyme. This observation accounts for the widely held view that avidin inhibition is irreversible.

As avidin has a very high affinity for biotin, it is a potent and highly specific inhibitor of biotin-dependent carboxylases. Although this has been known for over 20 years, there has been relatively little work on the kinetics or the mechanism of this inhibition. The usual practice is to exploit the specificity of avidin to provide evidence for biotin dependence but not to study the inhibition per se (e.g., Fall & Hector, 1977).

The inhibition by avidin of acetyl-CoA carboxylase (EC 6.4.1.2) has been studied by Moss & Lane (1972), but their results are complicated by changes in the state of polymerization of the enzyme. They concluded that acetyl-CoA carboxylase can exist as an active, avidin-insensitive, filamentous polymer and as an inactive, avidin-sensitive protomer. No attempt was made to measure the kinetics of formation of the avidin–protomer complex or its dissociation constant. Kaziro et al. (1960) studied the inactivation of propionyl-CoA carboxylase (EC 6.4.1.3) by avidin, and Green (1975) has used their data to calculate the rate constant for the formation of the enzyme–avidin complex. The most extensive study of avidin inhibition was reported by Scrutton & Utter (1965, 1967) working with pyruvate carboxylase (EC 6.4.1.1). They followed the development of inhibition and found that it was prevented by high concentrations of ATP and enhanced by acetyl-CoA. A curious feature of this work was the observation that the reaction order for the formation of the enzyme–avidin complex was 1.4 with respect to avidin concentration.

Partial reactivation of pyruvate carboxylase has been reported by Scrutton & Mildvan (1968), while Halenz et al. (1962) reported complete restoration of the propionyl-CoA–methylmalonyl-CoA exchange reaction catalyzed by propionyl-CoA carboxylase. However, most workers have considered the inhibition of biotin-dependent carboxylases to be irreversible, as complete reactivation has not been achieved for any of these enzymes [see Green (1975)].

The work of Scrutton & Utter (1965, 1967) indicates that binding of avidin to pyruvate carboxylase is a relatively slow process which occurs on a time scale of several minutes. The reason for the slow formation of the enzyme–avidin complex has not been elucidated, but recent advances in the theory [Cha, 1975; see also the correction, Cha (1976)] and analysis (Williams et al., 1979; Williams & Morrison, 1979) of slow-binding inhibitors suggested that the avidin–pyruvate carboxylase interaction is a system which is worthy of reinvestigation. In the present study we show that the combination of avidin with chicken liver pyruvate carboxylase is a simple second-order process with a rate constant of 1.42 × 10^5 M^{-1} s^{-1}. No evidence was adduced for a slow isomerization of the free enzyme or the enzyme–avidin complex. The inhibition by avidin can be partially reversed by biotin (rate constant = 1.45 × 10^{-5} s^{-1}), and the reason that full activity cannot be restored is that the enzyme–avidin complex is relatively unstable and undergoes an irreversible loss of activity at a rate which is 5 times the rate of avidin dissociation.

Experimental Procedures

Avidin and 2-(4′-hydroxyazobenzene)benzoic acid were obtained from Sigma Chemical Co., while [^{14}C]biotin was obtained from Amersham Australia. All other materials were high-purity preparations from commercial suppliers except for chicken liver pyruvate carboxylase which was purified as described elsewhere (Goss et al., 1979). The standard assay for pyruvate carboxylase was conducted at 30 °C in a final volume of 1 mL and contained 2.5 mM ATP, 7 mM $MgCl_2$, 10 mM pyruvate, 20 mM $NaHCO_3$, 0.25 mM acetyl-CoA, 0.24 mM NADH, and 11 units of malate dehydrogenase (EC 1.1.1.37) in 0.1 M Tris-HCl buffer, pH 7.8. The reaction was started by the addition of pyruvate carboxylase, and NADH oxidation was determined by following the absorbance at 340 nm. In experiments designed to study the onset of inhibition by avidin, the ATP concentration was reduced to 0.25 mM, assays contained up to 4.4 × 10^{-7} M avidin, and the enzyme was

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diluted in the presence of 0.25 mM acetyl-CoA before use. Throughout this report, concentrations of avidin and pyruvate carboxylase refer to those of the tetramers of both molecules unless specifically stated otherwise.

The reversal of avidin inhibition was studied by incubating 6–7 μM pyruvate carboxylase plus other additions as noted in the text with a 2- or 5-fold molar excess of avidin at 30 °C in 0.1 M Tris-HCl buffer, pH 7.2. After 20 or 30 min, the solution was diluted with an equal volume of 20 mM biotin in 0.1 M Tris-HCl buffer and incubation continued at 30 °C. At various time intervals, 10-μL samples were removed, and residual pyruvate carboxylase activity was determined by using the standard assay procedure.

The stoichiometry of binding between avidin and pyruvate carboxylase was determined by using the dye 2-(4′-hydroxyazobenzene)benzoic acid. A 10 mg/mL solution of avidin in 0.1 M Tris-HCl buffer (pH 7.2) was prepared, and the concentration of biotin-binding sites was determined by titration with [^{14}C]biotin (Rylatt et al., 1977). This method was also used to determine the biotin content of a sample of pyruvate carboxylase.

A solution containing 5 μM avidin sites and 50 μM dye in 0.1 M Tris-HCl buffer (pH 7.2) was prepared and 1 mL of this solution was placed in one compartment of each of two tandem cuvettes at 25 °C, while the second compartment of each cuvette contained 1 mL of a 5 μM solution of avidin sites. In the case of the sample cuvette, 2 μL of chicken liver pyruvate carboxylase (675 pmol of biotin) was added to the dye/avidin mixture while 2 μL of buffer was added to the avidin compartment. In the reference cuvette, enzyme was added to the avidin compartment and buffer to the dye/avidin compartment. The change in absorbance at 500 nm was monitored as the enzyme displaced the dye from avidin. When a stable absorbance had been attained, further 2-μL aliquots of enzyme and buffer were added in the same manner, and in this way a titration curve was obtained.

Toward the end of the titration, longer periods of time were required to achieve a stable absorbance and the end point for the titration was therefore determined by two alternative procedures. In the first procedure, 2 μL of 10 mM biotin was added at the end of the titration, thereby occupying any remaining avidin sites. In the second procedure, 2 μL of 10 mM biotin was added instead of the first addition of pyruvate carboxylase.

Theory and Data Analysis

Onset of Inhibition. When pyruvate carboxylase is added to a reaction mixture which contains avidin in addition to all of the substrates, the progress curve for the reaction shows a progressive increase of inhibition over a period of several minutes (Figure 1). This slow development of inhibition of enzymic activity may be explained in several different ways, and these are summarized in Figure 2. In mechanism A, the interconversion between E and EI is a slow process, and it is this feature which results in the slow development of inhibition. By contrast, mechanism B assumes that there is rapid formation of an EI complex which then slowly isomerizes to a second complex, EI*. As more enzyme is drawn into EI*, inhibition becomes progressively more pronounced. Finally, in mechanism C, the enzyme form (E) which combines with the substrate is unable to combine with inhibitor, while E′ can only bind the inhibitor. The slow conversion of E to E′ results in the slow development of inhibition.

Mechanisms A and B have been considered previously by Cha [1975; see also the correction, Cha (1976)]. He also discussed an enzyme isomerization model which differs from

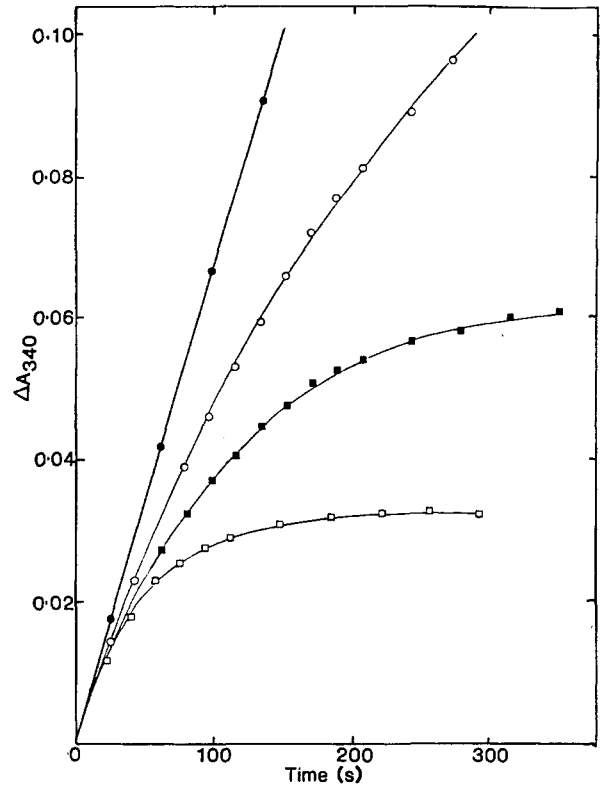


FIGURE 1: Onset of inhibition of chicken liver pyruvate carboxylase by avidin. Pyruvate carboxylase was assayed as described under Experimental Procedures by using avidin concentrations of 0 (●), 74 (○), 147 (■), or 294 nM (□). From each progress curve the absorbance at 340 nm was measured at several points along the curve. Equation 3 was fitted to the corrected absorbancies as described under Theory and Data Analysis, and the data were recalculated as the change in absorbance, relative to the estimated absorbance at zero time. The lines drawn represent the fitted curves.

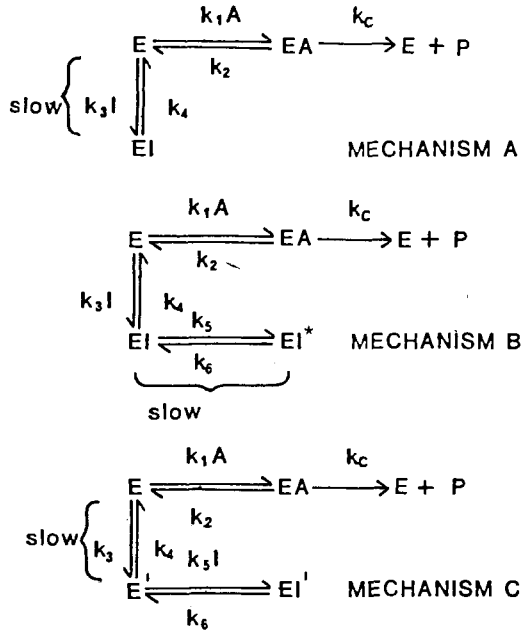


FIGURE 2: Possible mechanisms for slow-binding inhibition.

mechanism C in that the slow step was assumed to be the interconversion of E′ and EI′. This variant is not considered here as it is indistinguishable from mechanism A unless rapid reaction techniques are used. All three mechanisms are special cases of a general scheme which was introduced by Frieden (1970).

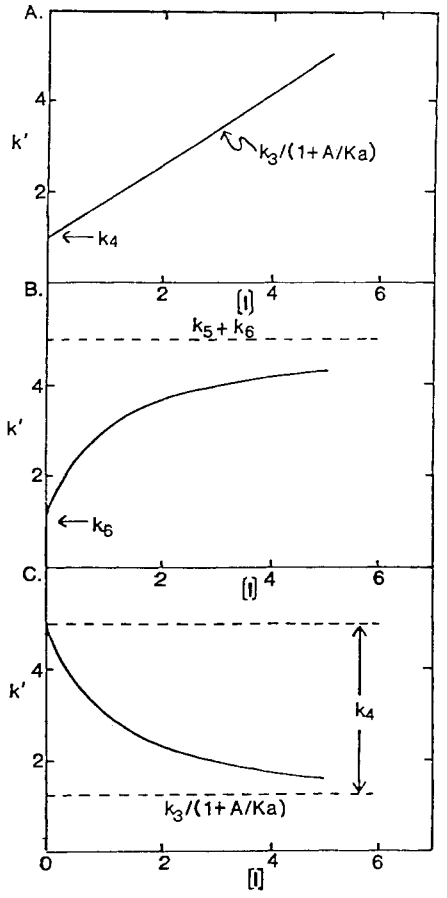


FIGURE 3: Diagnostic plots of k' vs. inhibitor concentration for the three mechanisms shown in Figure 2. Mechanism A predicts a linear relationship between k' and inhibitor concentration with a slope and intercept as shown in panel A. Mechanisms B and C predict hyperbolic relationships with the indicated intercept and asymptote as shown in panels B and C, respectively. The theoretical curves were calculated by using the following numerical values: (A) $k_3 = 1.6$, $k_4 = 1.0$; (B) $k_3/k_4 = 2.0$, $k_5 = 4.0$, $k_6 = 1.0$; (C) $k_3 = 2.0$, $k_4 = 4.0$, $K_i = 1.0$. In all cases, $A = K_a$.

For each of these mechanisms, the rate of catalysis (v) varies with time (t) according to

$$v = v_s + (v_0 - v_s) \exp(-k't) \quad (1)$$

where v_0 is the rate at $t = 0$, v_s is the rate as $t \rightarrow \infty$, and k' is an apparent first-order rate constant. The meaning of k' depends on the particular mechanism and is summarized in eq 2a, 2b, and 2c for mechanisms A, B, and C, respectively.

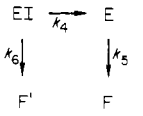
$$k' = \frac{k_4(1 + A/K_a + I/K_i)}{1 + A/K_a} \quad (2a)$$

$$k' = \frac{k_6(1 + A/K_a + I/K_i)}{1 + A/K_a + k_3I/k_4} \quad (2b)$$

$$k' = \frac{k_3}{1 + A/K_a} + \frac{k_4}{1 + I/K_i} \quad (2c)$$

where $K_i = k_4/k_3$ in eq 2a, $K_i = k_4k_6/[k_3(k_5 + k_6)]$ in eq 2b, and $K_i = k_6/k_5$ in eq 2c. In all cases, $K_a = (k_2 + k_c)/k_1$ and is the Michaelis constant for substrate A. The three mechanisms each produce a characteristic plot of k' vs. inhibitor concentration. Mechanism A predicts a linear relationship with a slope of $k_3/(1 + A/K_a)$ and an intercept on the ordinate of k_4 as shown in Figure 3A. Mechanisms B and C predict hyperbolic relationships but differ in that k' increases as I increases for mechanism B (Figure 3B) while mechanism C predicts an inverse relationship (Figure 3C).

Scheme I



Equation 1 describes the variation of the instantaneous reaction velocity with time. In practice, such velocities are difficult to measure from curves such as those depicted in Figure 1, and the preferred procedure is to integrate eq 1 to obtain the dependence of product formation (P) on time. Often the origin on the concentration axis will not be known with certainty; for example, the pyruvate carboxylase reaction is followed by coupling oxalacetate formation to NADH oxidation, which is monitored at 340 nm. However, the initial absorbance may not be known with absolute precision, and in these circumstances it is useful to introduce an additional parameter (d) which represents the initial absorbance. Incorporating this displacement term into the integrated form of eq 1, we get

$$P = v_st + (v_0 - v_s)[1 - \exp(-k't)]/k' + d \quad (3)$$

In the experiments described below (see Results) the usual procedure was to measure P at various time intervals to obtain 10–18 data points. These were corrected for the slow drift in absorbance in the absence of pyruvate carboxylase which may result from contamination of commercial malate dehydrogenase with traces of lactate dehydrogenase. These corrected data were then analyzed by fitting eq 3 to obtain values and standard errors for v_0, v_s, k', and d. Fitting was achieved by using a nonlinear regression program which was written in Fortran by R.G.D. This program calculates standard errors by matrix inversion, but spot checks using support planes and the jackknife method gave similar results (Duggleby, 1980). The lines in Figure 1 show the fitted curves at three avidin concentrations.

Cornish-Bowden (1975) has shown that relatively small systematic errors can have a substantial effect on parameters obtained from the analysis of progress curves. In the present case there are two potential sources of systematic error. The first results from the fact that the exact moment of enzyme addition may be uncertain and all t values will be displaced by an unknown amount. This, in turn, will affect the estimates of v_0 and d, but will not affect k' or v_s. The second potential source of systematic error is from the absorbance drift mentioned above. Slight inaccuracies in this value will have a relatively small effect and will be confined to the estimates of v_0 and v_s. Neither source of systematic error will affect the value of k', which is, therefore, the most reliable parameter for further analysis.

Reversal of Inhibition. It will be shown later that the interaction of avidin with chicken liver pyruvate carboxylase is described by mechanism A, with K_i being in the nanomolar range. Thus, if a pyruvate carboxylase solution in the micromolar concentration range is incubated with a small molar excess of avidin, then essentially all of the enzyme will be converted to the enzyme–avidin complex. If this is then diluted into a large excess of biotin, there will be a slow reversal of inhibition as avidin dissociates. The rate of dissociation is very low, and over the period of several hours which is involved in such an experiment, it was found that there is a substantial loss of enzymic activity in controls without avidin (Figure 4A). If we are to accept that free enzyme will undergo an irreversible inactivation, we must also admit the possibility that

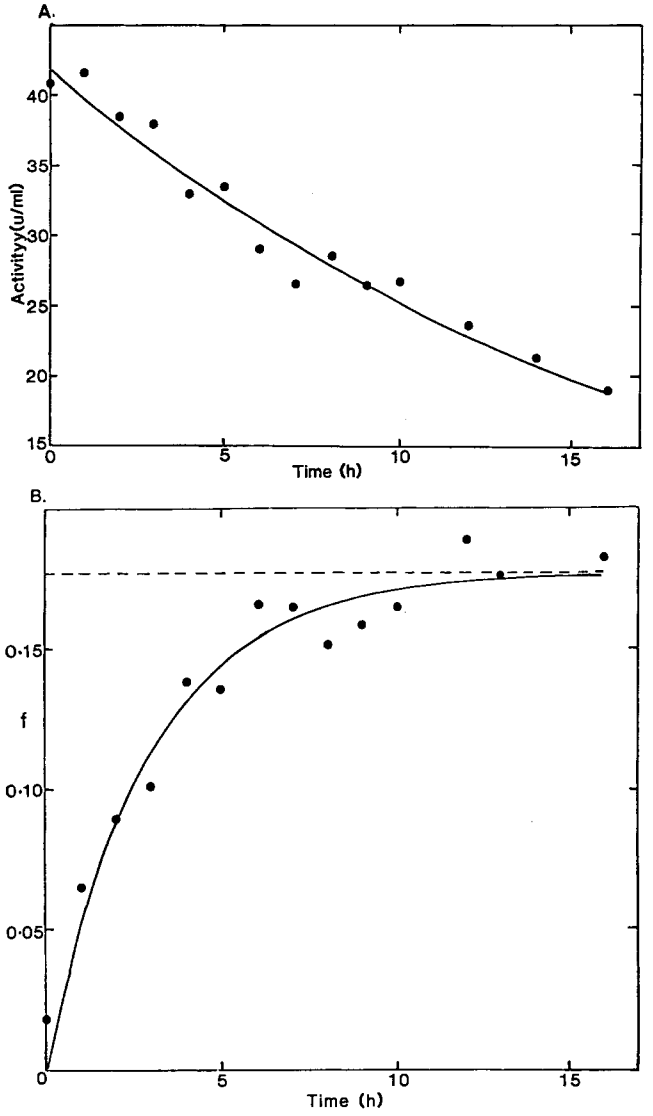


FIGURE 4: Reversal of avidin inhibition of chicken liver pyruvate carboxylase. Incubations were performed as described under Experimental Procedures and samples assayed at intervals for residual pyruvate carboxylase activity. Panel A shows the decline of enzymic activity in the absence of added avidin. These data represent the control for experiments 2 and 3 of Table I. The line is the best fit of eq 7 to the data. Panel B shows the recovery of enzymic activity on incubation of the enzyme–avidin complex with excess biotin. Results are expressed as the fraction of activity in the sample, compared to a control without added avidin. This experiment is that identified as experiment 3 in Table I. The solid line is the best fit of eq 8 to the data while the broken line is the theoretical asymptote.

the enzyme–avidin complex can undergo a similar decay. Thus we may formulate a model (see Scheme I) and write the differential equations

$$\frac{d[EI]}{dt} = \frac{dE_t}{dt} - (k_4 + k_6)[EI] \quad (4)$$

$$\frac{dE}{dt} = k_4[EI] - k_5E \quad (5)$$

These are readily integrated by using Laplace–Carson transforms to give an expression for the concentration of E as a function of time.

$$E = k_4E_t\{\exp(-k_5t) - \exp[-(q + k_5)t]\}/q \quad (6)$$

In eq 6, E_t is the total enzyme concentration, and q is equal

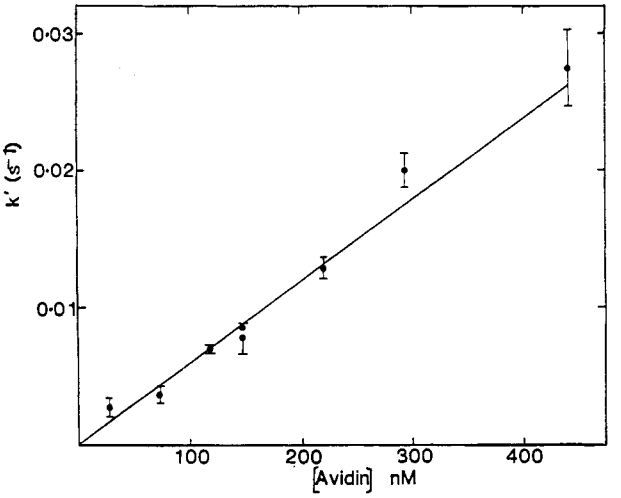


FIGURE 5: Dependence of k' of avidin concentration for chicken liver pyruvate carboxylase. Progress curves, such as those shown in Figure 1, were analyzed as described under Theory and Data Analysis to yield values for the apparent first-order rate constant, k'. These values are plotted against the concentration of avidin. The error bars are standard errors as obtained from fitting eq 3 to the progress curve data. The line represents the best fit obtained by weighted linear regression and is described by the equation $k' = (-1.5 \pm 6.0) \times 10^{-4}$ $s^{-1} + (6.01 \pm 0.44) \times 10^4$ M^{-1} s^{-1} ([avidin]).

to $k_4 - k_5 + k_6$. In the absence of avidin, no EI will be present initially and the concentration of E (which we shall denote as E^0) is given by

$$E^0 = E_t \exp(-k_5t) \quad (7)$$

If we assume that enzymic activity is proportional to the amount of E, we may define the fractional activity (f) at any time as $f = E/E^0$, and by substitution of eq 6 and 7 we get

$$f = k_4[1 - \exp(-qt)]/q \quad (8)$$

Equation 8 predicts that the fractional activity will rise from a starting value of zero to a stable plateau of k_4/q, and the experimental data conform to this prediction (Figure 4B).

Equation 7 was fitted to enzyme decay data of the type shown in Figure 4A by using a BASIC nonlinear regression program which has been described elsewhere (Duggleby, 1981) to obtain values for E_t and k_5. This computer program was also used to fit eq 8 to fractional activity data to determine values for k_4 and q.

Results

Onset of Inhibition. Addition of chicken liver pyruvate carboxylase to assay mixtures which contained between 0 and 441 nM avidin yielded a series of progress curves which showed marked downward curvature. Some examples are shown in Figure 1. These data were analyzed as described under Theory and Data Analysis to give values for v_0, v_s, k', and d at each inhibitor concentration. The values for k' are plotted against avidin concentration in Figure 5 from which it is seen that there is a linear dependence of k' on avidin concentration. This linear relationship is characteristic of mechanism A (cf. Figure 3A), and we can determine k_3 and k_4 from the slope and intercept of Figure 5. Weighted linear regression gave a slope of $(6.01 \pm 0.44) \times 10^4$ M^{-1} s^{-1} and an intercept of $(-1.5 \pm 6.0) \times 10^{-4}$ s^{-1}, the latter being an estimate of k_4.

Scrutton & Utter (1965) have shown that avidin binding is competitive with respect to ATP; thus, substrate A in mechanism A represents ATP. As is seen from Figure 3A,

Table I: Reversal of the Biotin Inhibition of Chicken Liver Pyruvate Carboxylase[a]

expt	pyruvate carboxylase (μM)	avidin (μM)	addition	k_4 (10^{-5} s^{-1})	q (10^{-5} s^{-1})	k_5 (10^{-5} s^{-1})	k_6 (10^{-5} s^{-1})
1	3.46	19.8	none	1.44 ± 0.26	11.25 ± 2.82	0.76 ± 0.30	10.57
2	3.33	16.7	none	1.02 ± 0.11	5.02 ± 0.84	1.40 ± 0.09[b]	5.40
3	3.33	6.7	none	1.65 ± 0.16	9.33 ± 1.10	1.40 ± 0.09[b]	9.08
4	3.46	19.8	0.2 mM DTE[c]	1.79 ± 0.23	9.42 ± 1.73	1.22 ± 0.20	8.85
5	3.33	16.7	0.2 mM DTE	1.40 ± 0.16	6.39 ± 1.03	1.54 ± 0.09[d]	6.53
6	3.33	6.7	0.2 mM DTE	1.88 ± 0.10	10.03 ± 0.68	1.54 ± 0.09[d]	9.69
7	3.33	16.7	0.25 mM acetyl-CoA	1.26 ± 0.11	6.57 ± 0.78	1.37 ± 0.12	6.68

[a] Chicken liver pyruvate carboxylase was incubated with avidin for 30 min (experiments 1 and 4) or 20 min (experiments 2, 3, 5, 6, and 7) and then diluted with an equal volume of 20 mM biotin as described under Experimental Procedures. Controls were treated in the same way except that avidin was omitted. The concentrations of enzyme, avidin, dithioerythritol, and acetyl-CoA are final concentrations after dilution. Residual enzymic activity was determined at various times after dilution, and eq 7 was fitted to the data from control incubations to determine the values of k_5. The results from incubations with avidin were converted to fractional activities relative to the corresponding control, and eq 8 was fitted to the resulting data to determine k_4 and q. Values for k_6 were calculated by using the relationship $k_6 = q + k_5 - k_4$. [b] The controls for experiments 2 and 3 were the same set of data. [c] DTE, dithioerythritol. [d] The controls for experiments 5 and 6 were the same set of data.

the slope of the plot of k' vs. inhibitor concentration is equal to $k_3/(1 + A/K_a)$, and it is necessary to determine K_a in order to calculate k_3. The Michaelis constant for ATP was found to be 164 ± 10 μM (data not shown). Combining this value with the known concentration of ATP in the assay (250 μM), we get $k_3 = (1.52 \pm 0.12) \times 10^5$ M^{-1} s^{-1} from the slope. Repetition of this experiment gave $k_3 = (1.33 \pm 0.11) \times 10^5$ M^{-1} s^{-1} and $k_4 = (5.9 \pm 4.2) \times 10^{-4}$ s^{-1}. There is good agreement between the two estimates of k_3 [weighted mean = $(1.42 \pm 0.09) \times 10^5$ M^{-1} s^{-1}], but k_4 is not well determined. The most that one can conclude with any degree of confidence is that k_4 is less than 2×10^{-3} s^{-1}, yielding a K_i of less than 14 nM.

If mechanism A is applicable to the interaction between an enzyme and an inhibitor, then v_0 should be independent of inhibitor concentration (Williams et al., 1979). We sought to verify this prediction by using the data obtained in the two experiments described above. The v_0 value obtained from the analysis of each progress curve is plotted against avidin concentration in Figure 6. In one experiment (closed circles) there appeared to be a slight downward trend, but this was not confirmed in the second experiment (open circles). As noted under Theory and Data Analysis the estimate of v_0 is vulnerable to systematic errors, and therefore, the values cannot be regarded as a very stringent test of mechanism A. The data in Figure 6 are consistent with the prediction that v_0 is unaffected by the avidin concentration.

Reversal of Inhibition. From the analysis of the onset of inhibition described above it was not possible to obtain a reliable value for k_4, the rate of dissociation of avidin from pyruvate carboxylase. For this reason we studied the dissociation directly by incubating the enzyme–inhibitor complex with a very large excess of biotin. Although some activity was recovered in this way, the analysis is not straightforward, as controls without avidin showed a substantial loss of activity over the period of 14–16 h which is necessary for the experiment (Figure 4A). The reactivation data were consistent with Scheme I and were analyzed by fitting eq 8 to obtain the values $k_4 = (1.44 \pm 0.26) \times 10^{-5}$ s^{-1} and $q = (1.13 \pm 0.28) \times 10^{-4}$ s^{-1}. From a control without avidin we may determine k_5 by fitting eq 7 to the data and obtain a value of $(7.59 \pm 2.98) \times 10^{-6}$ s^{-1}. Since k_4, k_5, and q are now known, we may also determine k_6 by using the relationship $k_6 = q + k_5 - k_4$ to obtain a value of 1.06×10^{-4} s^{-1}. These values are summarized in Table I as experiment 1. It is of interest that the rate constant for the change undergone by the enzyme–avidin complex (k_6) is 14 times greater than the corresponding rate

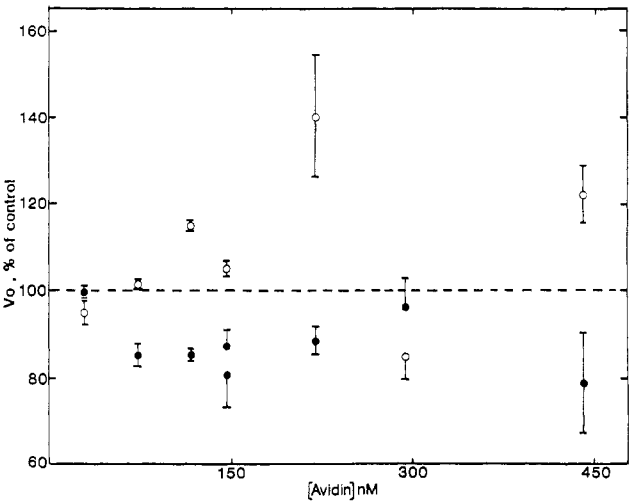


FIGURE 6: Dependence of the initial velocity on avidin concentration for chicken liver pyruvate carboxylase. Progress curves, such as those depicted in Figure 1, were analyzed as described under Theory and Data Analysis to yield values for the initial velocity, v_0. These values were expressed as a percentage of the velocity in the absence of avidin and plotted against avidin concentration. The error bars are standard errors as obtained from fitting eq 3 to the progress curve data. Open and closed symbols are from different series of experiments. The broken line is the theoretical relationship for mechanism A.

constant for the free enzyme (k_5). The instability of EI accounts for the fact that a rather small proportion of the initial activity can be recovered by treatment with biotin. It should be noted, however, that the instability of E and EI could have had no significant effect on the experiments where the onset of inhibition was followed. The half-time for inactivation of EI is in excess of 100 min whereas progress curves were followed for 10 min at most.

Experiment 1 was repeated with a similar avidin concentration (experiment 2, Table I) and with one-third the avidin concentration (experiment 3, Table I). The results from these experiments are essentially identical. We attempted to prevent enzyme inactivation by including either dithioerythritol (experiments 4–6, Table I) or acetyl-CoA (experiment 7, Table I), but these additions had no significant effect on the decay of free enzyme (k_5) or of the enzyme–avidin complex (k_6). The rate of dissociation of avidin (k_4) was also unaffected by these additions. The weighted mean value of k_4 from all experiments is $(1.45 \pm 0.13) \times 10^{-5}$ s^{-1}.

Stoichiometry of Binding. The stoichiometry of binding between avidin and pyruvate carboxylase was determined by

using the dye 2-(4′-hydroxyazobenzene)benzoic acid as described under Experimental Procedures. There was a linear relationship between the change in absorbance at 500 nm and the amount of enzyme added, with a slope of 0.0105 $A/\mu M$. The end point of the titration, determined by addition of biotin to a fresh solution of the dye/avidin mixture, was 0.053 A from which the stoichiometry of binding may be calculated at 1.01 mol of pyruvate carboxylase active sites/mol of avidin sites. The end point of the titration, determined by addition of biotin at the completion of the titration, was slightly higher and corresponds to a stoichiometry of 1.12.

Discussion

It has been known for many years that pyruvate carboxylase is inhibited by avidin (Utter & Keech, 1960), but the kinetics of this process have remained obscure. The very low K_i for avidin and its apparent irreversibility, coupled with the fact that inhibition develops slowly, invalidates the usual kinetic methods for studying inhibitors. Frieden (1970) recognized the existence of slow phenomena in enzyme-catalyzed reactions, and Cha [1975; see also the correction, Cha (1976)] has developed several models for slow-binding inhibitors. These models (Figure 2) include mechanism A, in which there is a slow interaction between the enzyme and the inhibitor, and mechanism B, in which there is rapid formation of an enzyme–inhibitor complex (EI) followed by a slow isomerization to a second complex, EI*. In the present work we have also considered a third case (mechanism C) in which there is a slow isomerization step prior to binding of the inhibitor. The analysis of slow-binding inhibitors has been discussed by Williams et al. (1979) and by Williams & Morrison (1979), and this aspect has been extended here. For all three mechanisms, progress curves are described by eq 3, but no consideration has been given previously to the effects of small systematic errors in the data on the parameters of this equation. It is shown here that the apparent first-order rate constant k' can be estimated most reliably from progress curves such as those depicted in Figure 1. Moreover, the relationship between k' and inhibitor concentration depends on the mechanism, and diagnostic plots for mechanisms A, B, and C are given (Figure 3).

The inhibition of chicken liver pyruvate carboxylase by avidin obeys mechanism A, as judged by the linear dependence of k' on avidin concentration (Figure 5). From the slope of this plot the rate constant for the formation of the EI complex was estimated as $(1.42 \pm 0.08) \times 10^5$ M^{-1} s^{-1}. This value is within the range reported by Green (1975) for other biotin-dependent carboxylases (10^4–10^6 M^{-1} s^{-1}) but is substantially higher than that reported by Scrutton & Utter (1967) for pyruvate carboxylase under similar conditions. However, Scrutton and Utter conducted their experiments in the presence of sulfate ions whereas the present data were obtained under essentially sulfate-free conditions. We have shown (unpublished data) that in the presence of 0.5 M sulfate ions, k_3 falls by 3 orders of magnitude to approximately 10^2 M^{-1} s^{-1}. A further difference between our results and a previous study of pyruvate carboxylase is that Scrutton & Utter (1965) reported a reaction order of 1.4 for the rate of formation of the EI complex with respect to avidin concentration. In an attempt to resolve this discrepancy, we analyzed the values for k' according to the equation $k' = a + b[\text{avidin}]^n$ and obtained values for n of 1.27 ± 0.14 and 0.91 ± 0.18 in two experiments. The weighted mean value of 1.14 ± 0.17 does not differ significantly from unity. In the reaction schemes depicted in Figure 2, E represents one active site or subunit of the enzyme. Thus, a kinetic order of unity implies that each subunit of avidin behaves as if it were an independent entity. Consequently each enzyme subunit will bind one tetramer of avidin, and hence, one pyruvate carboxylase tetramer forms a complex with four avidin tetramers.

The use of the dye 2-(4′-hydroxyazobenzene)benzoic acid to determine both avidin and biotin concentrations has been described by Green (1965). This dye binds to avidin with the resultant formation of a new absorption band at 500 nm, and this process can be reversed by the addition of 4 mol of biotin/mol of avidin. We have exploited this property to determine the stoichiometry of binding between avidin and pyruvate carboxylase. The results indicate a stoichiometry close to 1.0, implying that one enzyme subunit binds to one avidin subunit and that one pyruvate carboxylase tetramer binds to one avidin tetramer. This is in apparent conflict with the results obtained from the onset of inhibition data. However, a major difference between the two types of experiments is that in the onset experiments, the concentration of avidin tetramers is between 60 and 880 times that of pyruvate carboxylase tetramers. By contrast, in the titration experiments this ratio varies between 1.1 and 8. This may imply that the stoichiometry of binding of avidin to pyruvate carboxylase, and hence the nature of the complexes formed, is dependent upon the concentration of avidin relative to the enzyme. This suggestion is consistent with the observations of Scrutton & Mildvan (1968) on the sedimentation properties of such complexes and of Cohen et al. (1979) on the appearance in the electron microscope of the complexes formed when pyruvate carboxylase interacts with nonsaturating and saturating concentrations of avidin. We would stress that the complex formed in our titration experiments is not visualized as a single entity containing one pyruvate carboxylase tetramer and one avidin tetramer; rather, it is an aggregate in which several tetramers of each molecule are associated together in an extended network.

Both avidin and pyruvate carboxylase are relatively large molecules with molecular weights of 68 000 and 500 000, respectively. It is worth considering the extent to which diffusion alone will control the rate of interaction. Assuming diffusion coefficients of 6.6×10^{-7} cm^2 s^{-1} and 3.4×10^{-7} cm^2 s^{-1} for avidin and pyruvate carboxylase, respectively, and a reaction radius of 5×10^{-8} cm, the diffusion-controlled limit may be calculated (Alberty & Hammes, 1958) to be 1.7×10^8 M^{-1} s^{-1}. This value is almost certainly an overestimate, as it ignores steric factors which would be expected to limit the interaction. The measured rate constant is 3 orders of magnitude lower, which strongly suggests that the diffusion process itself is not rate limiting. That steric factors are important is evidenced by the observation that the rate of binding of avidin to free biotin (Green & Toms, 1973) is 500-fold faster than that to pyruvate carboxylase.

There have been no reports of complete reversal of avidin inhibition for pyruvate carboxylase or any other biotin-dependent enzyme. By incubation with excess biotin we were able to recover some activity (Figure 4B), and analysis of these data showed that there are three processes occurring simultaneously (Scheme I). The enzyme–avidin complex can either dissociate or decay to an inactive form, and the rate of dissociation is only one-fifth of the rate of decay. Once free enzyme is formed, it too can decay, although it is much more stable than the enzyme–avidin complex. From several experiments, the rate constant for dissociation was found to be $(1.45 \pm 0.13) \times 10^{-5}$ s^{-1}. Slow as it is, this rate is considerably faster than the rate of dissociation of free biotin (Green & Toms, 1973), indicating a substantial influence of the protein

moiety of pyruvate carboxylase. In principle, this value for k_4 should equal that determined from the onset of inhibition. The latter value was estimated to be less than 2×10^{-3} s^{-1}, which is consistent with the prediction.

It might be argued that the linear dependence of k' on avidin concentration is consistent with mechanism B and that the data in Figure 5 represent the lower end of a curve which would saturate at much higher concentrations of avidin. Such a proposal cannot be eliminated, but it implies that in eq 2b, $k_5 \gg k_4$ so that the proportion of the enzyme which is present as EI is extremely small. Thus the mechanism degenerates into a form which is equivalent to mechanism A. There may be, and probably are, several transient enzyme forms between E and EI, but these were not detected in the present study and should not be proposed in the absence of definitive evidence for their existence. Thus, we are inclined to accept mechanism A as an approximate description of the interaction between avidin and pyruvate carboxylase.

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Mechanism for Catalysis and Regulation of Adenosine 5′-Triphosphate Hydrolysis by Chloroplast Coupling Factor 1†

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ABSTRACT: A comprehensive mechanism is proposed for the hydrolysis of ATP and its regulation by chloroplast coupling factor 1. In this mechanism, a regulatory site is proposed to bind nucleotides very tightly. When ADP occupies this site, the enzyme is inactive, but when ATP binds to this site, the enzyme is active. Furthermore, the rate of nucleotide dissociation from the regulatory site is greatly enhanced during ATP hydrolysis. Steady-state kinetic studies of ATP hydrolysis and the inhibition of hydrolysis by 2′(3′)-(trinitrophenyl)-ADP and -ATP reveal positive cooperativity in substrate binding at low substrate concentrations that is enhanced by the inhibitors. In terms of the proposed mechanism, this is due to the displacement of substrate from the regulatory site by inhibitors. The binding of the inhibitors is characterized by a dissociation constant in the nanomolar range. The initial rate of dissociation of ADP from the regulatory site during ATP hydrolysis has a maximum rate constant of 0.09 s^{-1} and a Michaelis constant for ATP of about 80 μM, which is similar to the apparent Michaelis constant associated with ATP hydrolysis. The binding of the substrate 1,N^6-ethenoadenosine 5′-triphosphate to a specific site on the enzyme was studied by following the time course of fluorescence quenching with the stopped-flow method. The binding process can be characterized by a mechanism in which the substrate and enzyme rapidly form a complex that undergoes a relatively slow conformational change. The rate constant characterizing the conformational change is larger than the catalytic turnover number, indicating this binding reaction can be part of the catalytic mechanism. This two-step binding process can be readily incorporated into the general mechanism proposed for ATP hydrolysis. A diverse number of other observations in the literature also are consistent with the proposed mechanism.

The coupling factor 1 (CF_1)[1] is the portion of the ATP synthesizing complex of chloroplasts that contains the catalytic site. It can be isolated as a soluble protein and is composed of five different types of subunits [cf. Baird & Hammes (1979)]. While only membrane-bound enzyme can synthesize ATP, both membrane-bound enzyme and soluble CF_1 catalyze the reverse reaction, ATP hydrolysis. The synthesis and hy-

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; EDTA, ethylenediaminetetraacetic acid; TNP-ADP and TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate and 5′-triphosphate, respectively; ϵ-ADP and ϵ-ATP, 1,N^6-ethenoadenosine 5′-diphosphate and 5′-triphosphate, respectively; Tris, tris(hydroxymethyl)aminomethane.

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