APS Kinase from *Penicillium chrysogenum*

DISSOCIATION AND REASSOCIATION OF SUBUNITS AS THE BASIS OF THE REVERSIBLE HEAT INACTIVATION*

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Franco Renosto, Peter A. Seubert†, Paul Knudson‡, and Irwin H. Segel†

From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

Adenosine-5′-phosphosulfate (APS) kinase from *Penicillium chrysogenum*, loses catalytic activity at temperatures > approximately 40 °C. When the heat-inactivated enzyme is cooled to 30 °C or lower, activity is regained in a time-dependent process. At an intermediate temperature (e.g., 36 °C) an equilibrium between active and inactive forms can be demonstrated.

APS kinase from *P. chrysogenum* is a dimer (*M* = 57,000–60,000) composed of two apparently identical subunits. Three lines of evidence suggest that the reversible inactivation is a result of subunit dissociation and reassociation. (a) Inactivation is a first-order process. The half-time for inactivation at a given temperature is independent of the original enzyme concentration. Reactivation follows second-order kinetics. The half-time for reactivation for a given concentration is increased. However, MgATP and MgADP markedly stimulated the reactivation process.

(b) The equilibrium active/inactive ratio at 36 °C increases as the total initial enzyme concentration is increased. However, *K*ₐₖₛₐₚ at 5 mM MgATP and 36 °C calculated as ([inactive sites]/[active sites]) = 0.5 [active sites] is near-constant at about 1.7 × 10⁻⁸ M over a 10-fold concentration range of enzyme. (c) At 46 °C, the inactive *P. chrysogenum* enzyme (assayed after reactivation) elutes from a calibrated gel filtration column at a position corresponding to *M* = 33,000.

Substrates and products of the APS kinase reaction had no detectable effect on the rate of inactivation. However, MgATP and MgADP markedly stimulated the reactivation process (*K*ₐₖₛₐₚ = 3 × 10⁶ M⁻¹ s⁻¹ at 30 °C and 10 mM MgATP). *K*ₐₖₛₐₚ for reactivation was a nearly linear function of MgATP up to about 20 mM suggesting that the monomer has a very low affinity for the nucleotide compared to that of the native dimer. *K*ₐₖₛₐₚ at 36 °C increases as the MgATP concentration is increased.

The inactivation rate constant increased as the pH was decreased but no *pK*ₐ could be determined. The reactivation rate constant increased as the pH was increased. An apparent *pK*ₐ of 6.4 was estimated.

The sulfate-activating enzymes, ATP sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4) and APS¹ kinase (ATP:adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25) catalyze the first two steps, respectively, in the incorporation of inorganic sulfate into biological molecules by most bacteria, yeast, fungi, and animals. The reactions of sulfate activation are shown below.

\[
\text{SO}_4^{2-} + \text{ATP} \rightleftharpoons \text{APS} + \text{PP},
\]

\[
\text{APS} + \text{ATP} \rightarrow \text{PAPS} + \text{ADP}
\]

\[
\text{PP}_i + \text{H}_2\text{O} \rightarrow 2 \text{P}_i
\]

Animals use PAPS exclusively for sulfate ester biosynthesis (see Refs. 2 and 3 for a survey of sulfation reactions carried out by animal tissues). Bacteria, yeast, and filamentous fungi may also form sulfate esters but the major function of PAPS in these organisms is to serve as the substrate for assimilatory sulfate reduction leading to the sulfur amino acids. Higher plants and algae use APS rather than PAPS for this purpose but still contain APS kinase (perhaps to produce PAPS for use in sulfate ester biosynthesis or as an APS reservoir). In spite of the importance and wide distribution of APS kinase in nature, only the enzymes from the alga *Chlamydomonas reinhardtii* (1, 7, 8) and the fungus *Penicillium chrysogenum* (6) have received significant attention in recent years.

During our recent characterization of the near-homogeneous APS kinase of *P. chrysogenum* (6) we noted an unusual feature. When the enzyme was heated to 50 °C, catalytic activity was rapidly lost. But all the activity returned upon storing the heated enzyme for 24 h at 0 °C. In this present report, we provide evidence that the reversible heat inactivation phenomenon is a result of subunit dissociation and reassociation. We also present some non-clasical integrated rate equations which are useful for characterizing first-order and second-order inactivation or reactivation processes.

MATERIALS AND METHODS

Chemicals and Enzyme Purification—The general methods used in this present study were identical to those described earlier (6). The enzyme preparation used for the reported studies had a specific activity of 24.4 units per mg protein under standard assay conditions at 30 °C and was judged to be 90% pure by sodium dodecyl sulfate-

* The abbreviations used are: APS, adenosine 5′-phosphosulfate (5′-adenylylsulfate); PAPS, adenosine 3′-phosphate, 5′-phosphosulfate (3′-phospho-5′-adenylylsulfate or 3′-phosphoadenosine 5′-phosphosulfate).

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‡ Recipient of a President's Undergraduate Fellowship from the University of California, Davis.

† † To whom reprint requests should be sent.
Enzyme Inactivation

Activity Measurements—Unless otherwise indicated, the standard assay mixture (1 ml final volume) contained 5 mM MgATP (from a premixed stock solution containing 50 mM MgATP and 60 mM MgCl₂ in 0.5 M Tris base), 5.5 μM APS, 5 mM excess MgCl₂, 1 mM KC1, 0.5 mM NADH, 0.4 mM M, P-enolpyruvate, 35 units of pyruvate kinase, 50 units of lactate dehydrogenase, 70-150 mM ammonium sulfate (provided by the Sigma lactate dehydrogenase/pyruvate kinase mixture), 25-50 units of nucleoside P1, and 0.1 μl of the APS kinase solution (about 0.2 μg of the purified enzyme), all in 0.1 M Tris-Chl buffer, pH 8.0 (standard buffer). The reaction was started by adding APS or enzyme after 5 min of equilibration of other components to remove traces of ADP in the ATP. The decrease in NADH absorbance at 340 nm was followed on a Gilford 250 recording spectrophotometer with the chart recorder calibrated to give a full scale deflection of 0.02 absorbance units. Reaction rates were determined by measuring the slope of the recorder tracing and subtracting the background rate (recorder drift and non-APS kinase-dependent NADH oxidation). At 0.02 absorbance units, full scale = 100 divisions; the background rate at 30 °C was generally five divisions X min⁻¹ or less.

Assay medium. Incubation times at this low enzyme concentration were usually restricted to <15 min to avoid complications arising from irreversible inactivation.

Enzyme Inactivation and Reactivation—The loss of enzyme activity was measured by one of three methods. In method A (the classical procedure), the enzyme was preincubated under the desired inactivation or reactivation conditions and periodically, a known volume of the solution was transferred to the complete assay medium for a determination of residual or regained catalytic activity. Method B was an advantage in that the effect of an individual compound (e.g., MgATP, APS, PAPS) on inactivation or reactivation can be tested. A major disadvantage of this method, especially for inactivation studies, is that the enzyme concentration in the preincubation medium must be relatively high so that the volume of the withdrawn heated sample is small compared to that of the cooler assay medium. But at high enzyme concentrations, reactivation occurs very rapidly when the temperature is decreased. Consequently, method A often yielded an artificially high initial activity reading. The problem could be ameliorated somewhat by restricting the enzyme concentration to <25 μM X ml⁻¹ in the preincubation medium and by transferring aliquots to the assay medium with a pipette tip heated to the same temperature as the preincubation medium. Also, when feasible, the MgATP concentration in the assay medium was kept below 3 mM (MgATP stimulates reactivation).

In method B, inactivation and reactivation were studied in the complete assay medium while the APS kinase-catalyzed reaction was proceeding. For inactivation measurements, the enzyme was added directly to the preheated assay medium and activity followed for the desired time. For reactivation measurements, the enzyme was inactivated externally and an aliquot added to the assay medium with a heated pipette tip. By restricting the recorder range to 0.02 absorbance units full-scale and readjusting the pen periodically by closing the light slit, rates at any time could be easily measured. Because both MgATP and APS are continuously regenerated and all other components were present in great excess, the extent of the catalytic reaction was essentially unlimited. Method B has the advantage of providing multiple activity measurements on a single sample. The disadvantage is that the effect of a single compound on the inactivation or reactivation rate cannot be determined. Nevertheless, method B was routinely used for quantitative measurements. Method A or method C (see below) was employed to determine whether a particular compound by itself had an effect on inactivation or reactivation.

In method C, the enzyme was preincubated at near final concentration in the assay cuvette with the compound to be tested. After fixed periods of time, the remaining assay components were added and the activity (initial rate) measured.

RESULTS

Preliminary Observations of Reversible Heat Inactivation—Fig. 1 shows the effect of heating the P. chrysogenum APS kinase (20 μg X ml⁻¹) for 1 min at various temperatures. The remaining catalytic activity decreased sharply as the preincubation temperature was increased, an entirely expected result. Unexpectedly, the post-heating activity plateaued at about 15% of the original activity. Also unexpected was the complete recovery of activity when the heated enzyme was placed on ice for 20 h. Further experiments established that the plateau level depended on both the initial enzyme concentration and the ATP concentration in the assay medium (i.e., the ATP concentration to which the cooled enzyme was exposed just before the activity was recorded). Evidence presented below suggests that the plateau activity seen in Fig. 1 results from the rapid reactivation which occurs during the transfer of the concentrated enzyme from the hot preincubation medium to the cooler assay medium containing ATP.

Unless otherwise indicated, all subsequent measurements of the rates of inactivation and reactivation were conducted directly in the assay medium at an enzyme concentration of approximately 0.2 μg X ml⁻¹. Incubation times at this low enzyme concentration were usually restricted to <15 min to avoid complications arising from irreversible inactivation.

Kinetics of Inactivation (Reversible Phase) —Fig. 2 shows that enzyme activity is lost in a first-order process. The semi-log plot for inactivation at 44 °C is linear down to at least 4% remaining activity, effectively eliminating the possibility that the "inactive" enzyme possesses significant intrinsic activity. The Arrhenius activation energy, E₂₅, for the loss of activity (slope of the inset) is about 90,000 cal X mol⁻¹. Other activation energy constants are listed in Table I. All the values are in the range expected for enzyme inactivation (10).

Variations in the initial enzyme concentration, [E]₀, had no effect on the half-life for inactivation at 60 °C, as expected for a first-order process. The semi-log inactivation plots at different initial enzyme concentrations were parallel; a replot of the extrapolated zero-time activities versus [E]₀, was linear passing through the origin.

Effect of MgATP and Other Ligands on Reversible Inactivation—In an experiment similar to that shown in Fig. 2, the effect of changing the MgATP concentration on the rate of inactivation at 42 °C was determined. As shown in Fig. 3, the semi-log inactivation plots were parallel over a MgATP concentration range which straddled the Kₘ for MgATP (Fig. 3, inset). Thus, it appears that MgATP has no effect on the inactivation process. In a separate series of experiments, the
enzyme was preincubated with varied concentrations of APS at 42 °C. After specified periods, a solution containing ATP and the other components of the assay mixture was added and the activity recorded (method C). APS at concentrations up to 1 mM (approximately 700 $K_{\text{cal}}$) had no effect on the inactivation rate. MgADP and PAPS were also ineffective in protecting the enzyme.

**Kinetics of Reactivation**—When the progress of the reactivation reaction was plotted in semi-log form, the line was curved (ever decreasing slope). Moreover, the half-time for reactivation was not constant over a range of initial inactive enzyme concentrations. Reactivation was clearly not a first-order process thus eliminating simple unfolding and refolding of the polypeptide chain as the basis of the reversible transition. Plots of reciprocal [inactive sites] versus time at three different initial enzyme concentrations were linear and par-

![Fig. 2. Kinetics of enzyme inactivation at different temperatures. APS kinase (0.23 μg in 10 μl) was added to a standard 1.0-ml assay mixture at the indicated temperatures and the activity monitored for up to 14 min. Activity is shown in relative terms where 100% represents the extrapolated zero-time activity of the enzyme at the given temperature. Preliminary experiments established that the coupling enzymes were stable (or at least not limiting) over the assay conditions. APS kinase (0.23 pg in 10 gl) was added to a standard 1.0-ml assay mixture at the indicated temperatures and the activity monitored for up to 14 min. Activity is shown in relative terms where 100% represents the extrapolated zero-time activity of the enzyme at the given temperature. Preliminary experiments established that the coupling enzymes were stable (or at least not limiting) over the assay period. Inset: Arrhenius plot of the inactivation data ($k = 0.693/t_{1/2}$).

![Fig. 3. Inactivation of APS kinase at different concentrations of MgATP. Inset: replot of the vertical-axis intercept. The replot yields the $V_{\text{max}}$ and $K_{\text{M}}$ for MgATP at 42 °C and 5.3 μM APS (44 units × mg protein⁻¹ and 1.5 mM, respectively, compared to 29 units × mg protein⁻¹ and 1.5 mM at 30 °C).

![Fig. 4. Kinetics of reactivation at different initial enzyme concentrations. APS kinase (22.2 μg × ml⁻¹ of the preparation corresponding to 20 μg × ml⁻¹ of the homogeneous enzyme) was heated at 60 °C for 1 min in 50 mM potassium phosphate buffer, pH 7.5. Then 40-μl aliquots were transferred with a hot (50 °C) pipette tip to 1.0, 1.8, and 3.0 ml of standard reaction mixture (2 mM MgATP; 5.5 mM APS) at 30 °C. The increase in APS kinase activity was monitored for up to 15 min. The remaining heated enzyme solution was cooled and stored for 20 h at 0 °C in order to obtain the activity corresponding to 100% reactivation. The nanomolar concentration of inactive sites at any time during reactivation was calculated from $A = A_x - A$ where $A_x = A$ (activity (units × ml⁻¹) at any earlier time, and $A_x$ = total nanomolar concentration of sites assuming a subunit molecular weight of 30,000.

**TABLE I**

Activation energy constants for reversible heat inactivation of APS kinase (P. chrysochromum) Determined at pH 8 in the presence of 2.5 mM MgATP, 5 μM APS, and 5 mM excess Mg²⁺.

| Constanta | Valueb |
|-----------|--------|
| $k$ at 42 °C (315 K) | 2.50 × 10⁻⁷ s⁻¹ |
| $t_{1/2}$ at 42 °C | 277 s (4.5 min) |
| $E_a$ | 90,300 cal × mol⁻¹ |
| $\Delta H^\ddagger$ | 88,670 cal × mol⁻¹ |
| $\Delta G^\ddagger$ | 22,225 cal × mol⁻¹ |
| $T\Delta S^\ddagger$ at 42 °C | 67,445 cal × mol⁻¹ |
| $\Delta S^\ddagger$ | 214 e.u. |

*a $\Delta G^\ddagger$ was calculated from $\Delta G^\ddagger = -RT \ln (k/\kappa T)$ where $R$ is the gas constant (1.987 cal × mol⁻¹ × deg⁻¹), $T$ is the absolute temperature (K), $k$ is the first-order rate constant for the loss of activity, $\kappa$ is Planck's constant (6.62 × 10⁻³⁴ erg × s), and $\kappa$ is Boltzmann's constant (1.38 × 10⁻¹⁸ erg × deg⁻¹). $\Delta H^\ddagger$ was calculated from $\Delta H^\ddagger = E_a - RT$. $T\Delta S^\ddagger$ was calculated from $T\Delta S^\ddagger = \Delta H^\ddagger - \Delta G^\ddagger$.

b As shown later, inactivation is a consequence of enzyme dissociation (dimer → 2 monomer). Thus, the first-order rate constant for the inactivating process is numerically equal to one-half of $k$ for the loss of activity. Since $k$ appears in a logarithmic term, the activation energy constants for dissociation are only slightly smaller than those shown for activity loss.
Reversible Inactivation of APS Kinase

For a bimolecular and second order reaction of the type

\[ 2E + \text{MgATP} \rightarrow 2E \cdot \text{MgATP} \]

the calculated rate constant at 30 °C and 2 mM MgATP is \( 7 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1} \).

\[ k_{\text{cat}} \]

Effect of MgATP and Other Ligands on Reactivation—

MgATP had a dramatic effect on the rate of enzyme reactivation (Fig. 5). At 30 °C and 15 mM MgATP, \( k_{\text{app}} \) was 45 \( \times \) 10^3 \( \text{M}^{-1} \cdot \text{s}^{-1} \), or about 45-fold greater than the estimated rate constant for reactivation at 30 °C in the absence of MgATP. The \( k_{\text{app}} \) was a linear function of the MgATP concentration up to 15 mM (Fig. 5, inset), a result suggesting that the affinity of the inactive enzyme for MgATP is much lower than that of the native enzyme (see “Appendix”).

The reactivation time course data shown in Fig. 5 were gathered in complete assay medium while the enzyme catalyzed the normal reaction (method B). In another series of experiments, the heat-inactivated enzyme was preincubated for various periods of time with individual ligands and then assayed for activity by adding the remaining reaction components (methods A and C). APS alone at 1.1 \( \mu \text{M} \) to 2.7 mM had no effect on the rate of reactivation. Similarly, \( \text{Mg}^{2+} \) (5 mM) alone or PAPS (46 \( \mu \text{M} = 6 \, K_{\text{M}} \)) alone had no effect.

Effect of pH on Inactivation and Reactivation—The effect of pH on inactivation and reactivation was studied in an attempt to identify amino acid side chains involved in stabilizing the dimer. At 30 °C, the inactivation rate increased sharply below pH 6 (Fig. 6A) but the \( k_{\text{app}} \) versus pH plot had no inflection point or maximum down to pH 5.6. (Lower pH values could not be tested because the inactivation rate was too rapid.) At 42 °C, the inactivation rate increased sharply below pH 7 (Fig. 6B). Separate experiments confirmed that the inactivation was reversible: over 70% of the activity lost after 19 min at pH 5.2 and 30 °C was recovered within 25 min after the pH was readjusted to 8.0. Fig. 6C shows the effect of pH on the \( k_{\text{app}} \) for reactivation. The reactivation rate constant was near-maximal at pH 8 and half-maximal at about pH 6.6 (pH values higher than 8.0 were not tested because nuclease P1 lost activity too rapidly). Given that the reactivation reaction is second order and assuming that (a) only one prototropic group on each subunit is involved (the same chemical entity on both) and (b) only the deprotonated subunit dimerizes, \( k_{\text{app}} \) is given by (see “Appendix”):

\[ k_{\text{app}} = \frac{k}{[H^+]^2} \]

Thus, \( k_{\text{app}} \) will equal 0.25 \( k \) (i.e. one-fourth \( k_{\text{max}} \)) when pH \( = \) pK\(_a\). Estimating \( k \) as 2.6 \( \times \) 10^3 \( \text{M}^{-1} \cdot \text{s}^{-1} \) (Fig. 6C), the apparent pK\(_a\) of the prototropic group is about 6.4.

\[ k = \frac{k_{\text{max}} \cdot [E]_0}{[E]_0 + K_{\text{M}}} \]

Demonstration of an Equilibrium between Active and Inactive Forms of the Enzyme—The experiments described above were performed at selected temperatures where the transition
under study goes essentially to completion \((T = 42 \, ^\circ C \text{ for inactivation}; \; T \leq 30 \, ^\circ C \text{ for reactivation})\). At an intermediate temperature \((36 \, ^\circ C)\) it was possible to demonstrate that the active \(\rightarrow\) inactive transition is an equilibrium process. Prolonged incubation of the enzyme at \(36 \, ^\circ C\) resulted in a loss of activity down to a limit of about 36\% of the zero-time value. Prolonged incubation at \(36 \, ^\circ C\) after inactivating the enzyme at \(60 \, ^\circ C\) resulted in an increase in activity up to about 30\% of the original, unheated activity (Fig. 7). Both experiments were performed at the same MgATP and enzyme concentrations. Similar experiments showed that the equilibrium activity at \(36 \, ^\circ C\) (approached from either direction) increased with increasing MgATP or enzyme concentration (Table II). In all cases, the reactivation curve plateaued at a slightly lower activity at \(36 \, ^\circ C\) (approached from either direction) increased with increasing MgATP or enzyme concentration (Table II). In all cases, the reactivation curve plateaued at a slightly lower activity at \(60 \, ^\circ C\) resulted in an increase in activity up to about 50\%, ascribed to the greater degree of irreversible inactivation of the enzyme with the latter becoming proportionately more significant as the enzyme concentration is decreased. Thus, the apparent \(k_{\text{eq}}\) values listed in Table II are probably slightly high.

Irreversible Inactivation—APS kinase is not indestructible. Prolonged storage of the enzyme at low concentration results in a slow irreversible loss of activity even at low temperatures. For example, at 0.2 \(\mu g \times ml^{-1}\), 30 \(^\circ C\), and pH 8.0 in the presence of 5 mM MgATP (and all other assay components except APS) about 2.5\% of the original activity is lost in 1 h. The enzyme could also be irreversibly inactivated at high concentrations by prolonged heating at high temperatures. For example, at 23 \(\mu g \times ml^{-1}\), the half-time for irreversible inactivation at \(80 \, ^\circ C\), pH 8.0, was 47 min (data not shown).

![FIG. 7. Demonstration that the active-inactive transition is an equilibrium process. A, inactivation of APS kinase at \(36 \, ^\circ C\). Exactly 100 \(\mu l\) of enzyme solution (2.3 \(\mu g \times ml^{-1}\)) at 0 \(^\circ C\) were transferred to 0.9 ml of standard assay medium at \(36 \, ^\circ C\) containing all reaction components except APS. After the desired preincubation time, APS was added to start the reaction (method C). Enzyme activity was measured immediately and for several minutes thereafter (as in method B). The experiment was repeated several times with different preincubation periods to generate the complete curve. B, reactivation of APS kinase at \(36 \, ^\circ C\). The enzyme (2.3 \(\mu g \times ml^{-1}\)) was heated at 60 \(^\circ C\) for 1 min and then 100 \(\mu l\) of the solution was transferred with a heated pipette tip to 0.9 ml of standard assay medium at \(36 \, ^\circ C\) containing all reaction components except APS. After the desired preincubation time, APS was added to start the reaction (method C). Enzyme activity was measured immediately and for several minutes thereafter (as in method B). The experiment was repeated several times with different preincubation periods to generate the complete curve.]

### Table II

| Variable \([\text{MgATP}]^2 \) (mM) | Activity at equilibrium | Apparent \(k_{\text{eq}}\) | Inactivation | Reactivation | \(0.5 \times \text{[Active sites]}^2\) |
|---|---|---|---|---|---|
| 1 | 26 | 16 | 5.0 \(\times\) \(10^{-8}\) | 1 | 2 |
| 2 | 30 | 21.5 | 3.5 \(\times\) \(10^{-8}\) | 2 | 3 |
| 3 | 37 | 30 | 2.0 \(\times\) \(10^{-8}\) | 3 | 4 |
| 4 | 42 | 45 | 1.0 \(\times\) \(10^{-8}\) | 4 | 5 |
| 5 | 50 | 52 | 0.5 \(\times\) \(10^{-8}\) | 5 | 6 |

* \(k_{\text{eq}}\) calculated from the average of the values listed in the second and third columns. The calculation is equivalent to \(K_{\text{eq}} = [\text{mominer}]^2/[\text{dimer}]\) (see text and Footnote).  
* The enzyme concentration was 0.23 \(\mu g \times ml^{-1}\) when MgATP was the variable.  
* Estimated from the earliest points.  
* Calculated as \(k_{\text{reactant}}/k_{\text{inact}}\).  
* The MgATP concentration was 5 mM when the enzyme concentration was the variable.  
* Concentration of sites (subunit concentration) calculated on the basis of 90\% purity and a subunit \(M_r = 30,000\).

Molecular Weight Estimates of the Native and Reversibly Inactivated Enzyme—At 22 \(^\circ C\), the \(P.\ chrysogenum\) APS kinase elutes from a calibrated Sephadex G-100 column between bovine serum albumin \((M_r = 67,000)\) and ovalbumin \((M_r = 43,000)\) at a position corresponding to a molecular weight of 57,000. When the column was run at 46 \(^\circ C\), no APS kinase activity was found in the eluate. After incubating the eluates overnight at 22 \(^\circ C\), APS kinase activity appeared in the fractions between ovalbumin \((M_r = 43,000)\) and chymotrypsinogen A \((M_r = 25,000)\) at a position corresponding to a molecular weight of 33,000.

When a partially purified preparation of APS kinase from \(P.\ duponti\) was passed through the gel filtration column, the enzyme eluted at the same position (relative to standards) at 22 and 44 \(^\circ C\). \((P.\ duponti\) is a thermophile and grows at temperatures up to about 50 \(^\circ C\).) Co-filtration of the \(P.\ chrysogenum\) and \(P.\ duponti\) enzymes at 22 \(^\circ C\) yielded a single broad activity peak. The band could be resolved into two overlapping peaks by assaying at 25 \(^\circ C\) (where the activity of the \(P.\ duponti\) enzyme was extremely low) and 45 \(^\circ C\) (after inactivation of the \(P.\ chrysogenum\) enzyme). At 44 \(^\circ C\), the \(P.\ chrysogenum\) enzyme was inactivated and eluted after the active \(P.\ duponti\) enzyme.

Spectral Changes—Heat treatment produced only small changes in the UV absorption spectrum of the enzyme. At 30 \(^\circ C\), \(\lambda_{\text{max}}\) was 279 nm. After 10 min at 42 \(^\circ C\), \(\lambda_{\text{max}}\) shifted to 277 nm and the absorbance at \(\lambda_{\text{max}}\) increased by about 6\%.

**DISCUSSION**

APS kinase from \(P.\ chrysogenum\) undergoes a reversible temperature and pH-dependent transition between an active form (at low temperature and high pH) and an inactive form (at high temperature and low pH). Inactivation follows first-order kinetics but reactivation follows second-order kinetics suggesting that the reversible transition involves dissociation and reassociation. The kinetics alone did not disclose the identity of the dissociable entity. There were, however, only
two possible scenarios. One is that high temperature induces the release of a low molecular weight stabilizer (e.g. nucleotide, metal ion) and low temperature promotes reassociation. The second is that the enzyme, which is a homodimer, dissociates into its subunits at high temperatures and reassociates at low temperature. The first possibility was excluded by the results of the gel filtration experiments. A low molecular weight stabilizer would have separated from the protein upon gel filtration at 46 °C. Yet, the enzyme reactivated completely after gel filtration under inactivating conditions. Furthermore, the inactive protein eluted at a position corresponding to a molecular weight of 33,000, which is close to half of the native molecular weight of 57,000. (If the native dimer is spherical but the individual subunit is not, the subunit would elute from the molecular weight calibrated column at a position corresponding to a molecular weight greater than 57,000/2). The cumulative results strongly support subunit dissociation and reassociation as the basis of the reversible transition:

\[ E_2 \text{ (inactive)} \]

High temp, low pH, low protein conc (fast) \[ \xrightarrow{2E} \text{ (inactive)} \]

Low temp, high pH, high protein conc (fast) \[ \xrightarrow{E} \text{ (denatured)} \]

The apparent equilibrium constants (dissociation constants) in the above scheme are defined as follows:

\[ K_D = \frac{[E_{\text{net}}]}{[E_{\text{active}}]} = \frac{k_{-1}}{k_1} \quad \text{and} \quad K_A = \frac{[E_{\text{active}}]}{[E_{\text{active}}]} = \frac{k_{-2}}{k_2} \]

\[ \alpha K_A = \frac{[E_{\text{active}}]}{[E]} = \frac{k_{-3}}{k_3} \]

\[ K_{\text{dissoc}} = \frac{[E_{\text{net}}]}{[E_{\text{active}}]} = \frac{k_{-4}}{k_4} \]

The relationship between MgATP binding and subunit interaction can be depicted as a simple random equilibrium if certain simplifying assumptions are made (see “Appendix”).

\[ \text{SCHEME 1} \]

The horizontal reactions are also second-order and hence, are highly concentration-dependent. At intermediary temperatures it is possible to demonstrate the composite \( E_{\text{active}} \overset{\alpha}{=} E_{\text{active}} \) equilibrium. For example, at 36 °C and 5 mM MgATP, the apparent equilibrium constant of the dimer \( = \) 2 monomer reaction is about \( 1.7 \times 10^{-4} \) M.

MgATP binds to the native enzyme with a \( K_a \) of 1.5 mM (6), whereas \( \alpha K_A \) appears to be \( \gg K_a \) (i.e. \( \alpha \gg 1 \)). In effect, protein association includes a conformational change in each subunit which increases the affinity of the active site for MgATP. Stated equivalently, the binding of MgATP to a free subunit promotes a conformational change which promotes association with another subunit. An alternative explanation, that MgATP binds only to the active dimer and pulls the association reaction to the left, can be discounted because at 30 °C, the equilibrium is far in favor of the active dimer even in the absence of MgATP. (If there is no back reaction of dimer \( \rightarrow \) 2 monomer at 30 °C, MgATP binding to the dimer cannot influence the subunit association reaction.) If the model shown in Scheme 1 is a reasonable approximation of the reversible inactivation phenomenon, we can conclude that \( k_a = k_i \) (at least at \( T > 40 °C \)), but \( k_i = k_i/a \gg k_4 \) (at least at \( T \leq 30 °C \)).

The pH profile for reactivation suggests that a prototropic group with a \( pK_a \) of about 6.4 must be deprotonated for subunit association to occur. There are three potential possible locations of this group. (a) It may reside at the subunit-subunit interface (i.e. it may play a structural role), (b) it may reside at the MgATP binding site, or (c) on MgATP itself. In possibilities b and c, the effect of pH would be indirect, \( k_{\text{in}} \) would increase with increasing pH because MgATP binding is increased. No conclusions can be drawn because we cannot directly determine the effect of pH on MgATP binding to the inactive subunit. However, kinetic experiments with the native enzyme showed no substantial change in the \( K_a \) between pH 6.4 and 8.0 (\( K_a = 1.25-1.5 \) mM; \( V_{\text{max}} \) at pH 6.4 is 60% that at pH 8.0).

Our observations prompt an old question: why do so many enzymes exist as oligomers when there is no discernible interaction between sites, and the separated subunits are structurally stable? One possible answer is that the folding of the polypeptide chain into the “best possible” tertiary structure is seldom sufficient to form an active site which combines high catalytic activity with high affinity for the substrates. Additional forces are required to further mold the active site into a functional entity. Association of monomers provides the final tailoring. This idea certainly seems to fit APS kinase whose subunit-monomer is highly stable but has a poor affinity for MgATP and no detectable catalytic activity.

The ease with which APS kinase undergoes reversible dissociation raises another question: is there any physiological function to the transition? Perhaps heat or low pH promote an inactivation process in vitro which mimics a metabolite-controlled process in vivo at normal growth temperature (approximately 10–35 °C for P. chrysochrome) and internal pH (probably > 7.0 for fungi). APS kinase is the second enzyme in the sulfate assimilation pathway but it catalyzes the first physiologically irreversible step. Thus, the APS kinase reaction is a good candidate for a regulatory site. The first reaction of the pathway (that catalyzed by ATP sulfurylase) has a \( K_{\text{eq}} \) of approximately \( 10^{-10} \) and thus, is likely to be at equilibrium at all times in vivo. (Equilibrium reactions cannot serve as control points in a multistep sequence.) Except for the report of Schwenn and Schlie showing that thioredoxin activates APS kinase from C. reinhardii (8), there is no information available concerning the regulation of sulfate activation in

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P. A. Seubert and I. H. Segel, unpublished results.
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Eucaryotic organisms. Experiments to identify potential effectors of the fungal APS kinase are in progress.

APPENDIX

Integrated Rate Equations for Some First-order and Second-order Enzyme Inactivation (or Reactivation) Processes

The rate equations derived below were considered in our analysis of the active \( \Rightarrow \) inactive transition of APS kinase.

Thermal or Chemical Inactivation in the Presence of a Partially Protective Ligand—A bound ligand may diminish the rate of inactivation but not be able to completely protect against the loss of activity. This is the usual situation for thermal inactivation and may also apply to chemical inactivations where the susceptible R group is not at the active site. The relevant reactions are shown below where \( k_1 \) and \( k_2 \) are first-order or pseudo first-order rate constants (see also Refs. 5 and 9).

\[
E \stackrel{k_1}{\longrightarrow} E' \quad \text{(inactive)}
\]

\[
A \quad K_A \quad k_3 = \beta k_h \quad E' A \quad \text{(inactive)}
\]

SCHEME 1

The rate of inactivation is given by:

\[
\frac{-d[E]}{dt} = k_1[E] + k_3[EA]
\] (1)

In order to integrate Equation 1 to obtain an expression for the activity remaining after any given time, \([E]\) and \([EA]\) must be expressed in terms of \([E]\), (total active enzyme concentration). The general procedure is as follows. Given that:

\[
\frac{[E]}{[E]_o} = \frac{[E]}{[E]} + \frac{[EA]}{[EA]}
\]

and by definition:

\[
\frac{[E]}{[EA]} = K_A \quad \text{or} \quad \frac{[EA]}{[E]} = \frac{[A]}{K_A}
\]

substitution yields:

\[
\frac{[E]}{[E]_o} = \frac{[E]}{[E]_o + \frac{[A]}{K_A}} = \frac{1}{1 + \frac{[A]}{K_A}}
\]

or

\[
[E] = \frac{[E]_o}{1 + \frac{[A]}{K_A}}
\]

similarly:

\[
\frac{[EA]}{[E]_o} = \frac{[A]}{k_3[K_A]} \quad \text{or} \quad \frac{[EA]}{[E]} = \frac{[E]_o}{K_A} \frac{[A]}{1 + \frac{[A]}{K_A}}
\]

Substituting for \([E]\) and \([EA]\) in Equation 1, we obtain

\[
\frac{-d[E]}{dt} = k_{app} \quad \text{or, upon integration:} \quad \ln \frac{[E]}{[E]_o} = \frac{k_{app}}{k_{app}} t
\] (2)

where:

\[
k_{app} = \frac{k_1}{1 + \frac{[A]}{K_A}} + \frac{k_3}{1 + \frac{K_A}{[A]}}
\] (3)

Note that if \( k_3 = 0 \), Equation 3 reduces to that for a completely protective ligand (4).

Equation 3 can also be written as:

\[
k_{app} = \frac{k_1}{1 + \frac{[A]}{K_A}}
\] (4)

Equation 4 was obtained by expressing \( k_3 \) in terms of \( k_1 \) (i.e. \( k_3 = @k_1 \)) and combining the two right-hand terms of Equation 3.

If \( A \) protects against inactivation, \( @ \) will be < 1. The \( k_{app} \) has limits of \( k_1 \) (at zero [A]) and \( @k_1 \) (at saturating [A]). But it is unnecessary to perform an experiment at saturating [A] to obtain \( @ \). The experimental procedure is to determine \( k_{app} \) at several different concentrations of [A] and then replot the data as \( \frac{l}{[A]} \) versus \( \frac{1}{[A]} \) where:

\[
[A] = k_{app} - k_{app} = \text{the difference between } k_{app} \text{ at } [A] = 0 \text{ and } k_{app} \text{ at an unsaturating [A]}
\]

(For chemical inactivations, \( k_{app} \) must be corrected at each [A] for any reagent-independent component.) The replot, which is based on the linear Equation 5,

\[
\frac{1}{\Delta k} = \frac{k_A}{k_{1}(1 - \beta) [A]} + \frac{1}{k_{1}(1 - \beta)}
\] (5)

has vertical and horizontal intercepts of \( 1/k_{1}(1 - \beta) \) and \(-1/K_A \), respectively. With \( k_1 \) known (from experiments without \( A \)), \( \beta \) can be calculated. There may be occasions when it is more convenient to replot the data as \( 1/\Delta k \) versus \( 1/[A] \) where: \( \Delta k = k_{app} - k_{app} = \text{the difference between } k_{app} \text{ at [A] = 0 and } k_{app} \text{ at an unsaturating [A]. (For chemical inactivations, } k_{app} \text{ must be corrected at each [A] for any reagent-independent component.) The replot, which is based on Equation 6,

\[
\frac{1}{\Delta k} = \frac{k_A}{k_{1}(1 - \beta) [A]} + \frac{1}{k_{1}(1 - \beta)}
\] (6)

has intercepts of \( 1/k_{1}(1 - \beta) \) and \(-1/K_A \). With \( k_1 \) known, \( k_1 \) can be calculated from the vertical-axis intercept.

The reaction scheme shown above could also describe the effect of a reversibly-bound ligand which renders the enzyme more susceptible to thermal or chemical inactivation, i.e. \( \beta > 1 \). In this case, the equations for the replots are:

\[
\frac{1}{\Delta k} = \frac{k_A}{(k_1 - 1) [A]} + \frac{1}{k_1} \quad \text{and}
\]

\[
\frac{1}{\Delta k} = \frac{k_A}{k_1 k_3(\beta - 1) [A]} + \frac{1}{k_1 k_3(\beta - 1)}
\] (7)

where:

\( \Delta k = k_{app} - k_{1} \)

and

\[
\Delta k = k_3 k_1 - k_{app}
\] (8)

The treatment described above is essentially identical to that used to analyze the effects of partial (hyperbolic) inhibitors and nonessential activators in steady-state kinetic experiments (Chapters 4 and 5 of Ref. 10), except that rate constants
rather than initial velocities are measured.

**Inactivation of Oligomeric Enzymes**—The oligomeric structure of most enzymes poses potential problems in analyzing the kinetics of inactivation. Even if ligand binding is normally Michaelian (i.e. the native enzyme displays neither positive nor negative cooperativity), inactivation may not follow simple first-order kinetics. For example, the semi-log plots will be curved (decreasing slope) if, as inactivation proceeds, the loss of a subunit decreases the rate of inactivation of remaining subunits, or decreases $K_A$ for a protective ligand. Inactivation events which render the remaining subunits more labile may or may not be detected; the semi-log plots may appear to be linear, or show a lag.

The fact that so many enzymes appear to follow first-order inactivation kinetics suggests that the potential complications stemming from oligomerization are either uncommon or are masked. For example, thermal inactivation of a single subunit of a dimer or tetramer may alter the stability of the oligomer sufficiently to cause simultaneous inactivation of the remaining subunits, i.e. a single "hit" is sufficient to cause the loss of all the catalytic sites of the native enzyme. On the other hand, chemical modification of an active site or structural residue may cause only a small change in the tertiary structure of a subunit, enough to eliminate catalytic activity in that subunit, but not enough to transmit instability to the unmodified subunits, or to alter the kinetic properties of the remaining active sites. An affinity label, which resembles a normal substrate sufficiently to be bound at an active site, is unlikely to alter the stability or kinetic properties of unoccupied sites on other subunits.

To illustrate how a seemingly complex situation can reduce to one that is easily analyzed, consider the thermal or chemical inactivation of a homodimer in the presence of a protective ligand which binds noncooperatively. The model, shown in Scheme II, assumes that a single hit is sufficient to inactivate both subunits. (The inactivation event could, for example, be the dissociation of the dimer into noncatalytic subunits.) It is further assumed that each enzyme species ($E_2$, $AE_2$, $E_2A$, and $AE_2A$) inactivates with a unique rate constant.

The $k_{app}$ for loss of activity will be numerically equal to twice the $k_{app}$ for dimer dissociation (one dissociation event loses two active sites). While $k_{app}$ is a complex function of $[A]$, the semi-log plots are linear at each $[A]$ regardless of the values of $\alpha$ and $\beta$. If $ak$ (the rate constant for the dissociation of singly occupied dimers) is the average of $k$ and $\beta k$, i.e. $\alpha = (1 + \beta)/2$, $k_{app}$ reduces to:

$$k_{app} = \frac{k(1 + \beta[A])}{K_A} \left(1 + \frac{[A]}{K_A}\right)$$

Equation 11 is identical to Equation 4 which was derived without regard to the oligomeric state of the enzyme.

**Effect of a Reversibly Bound Ligand on the Rate of Subunit Reassociation**—The general scheme for the association of two monomers or subunits in the presence of a reversibly bound ligand (including H$^+$) is shown below:

The differential rate equation is:

$$\frac{-d[E]}{dt} = k[E]^2 + 2ak[E] + \beta k[E]^2$$

where:

$$[E] = [E_2] + [E_1]$$

In Scheme III, $k$, $ak$, and $\beta k$ are the intrinsic rate constants for the productive collision of one subunit with another. The factor of 2 for the interaction of $E$ with $EA$ accounts for the increased probability of heterologous collisions. To illustrate the requirement for the additional factor, consider a system when $A$ has no effect on dimerization (i.e. $k = ak = \beta k$). Let $k = ak = \beta k = 1$, $[E_2] = 1$, and $-d[E]/dt = 1$ at all $[A]$. For example, at $[A] = 0$, $-d[E]/dt = k[E]^2 = 1 = 1$. At saturating $[A]$, $-d[E]/dt = \beta k[E]^2 = 1 = 1$. When $[A] = K_A$, $[E] = 0.5$ and $[EA] = 0.5$. Thus, $-d[E]/dt = k[E]^2 + k'[EA][EA] + \beta k[E]^2 = (1/0.5)^2 + k'(0.5)(0.5) + (1/0.5)(0.5) = 1$ where $k'$ is the effective rate constant for the interaction of $E$ with $EA$. In order for $-d[E]/dt$ to equal 1, $k'$ must equal 2. If $\alpha = 1$ and $k = 1$, then $k'$ must equal $2ak$.

If $E$, $A$, and $EA$ remain at equilibrium throughout the time course of dimerization (a reasonable assumption if $A$ binding and dissociation is faster than dimer formation), the concentrations of $E$ and $EA$ can be expressed in terms of $[E]$. As shown earlier:

$$[E] = \frac{[E_2]}{1 + \frac{[A]}{K_A}}$$

and

$$[EA] = \frac{[E_1]}{1 + \frac{[A]}{K_A}}$$

**Scheme II**

In the above scheme, $K_A$ represents the intrinsic $A$ dissociation constant of a site. The loss of active dimer is given by:

$$\frac{-d[E]}{dt} = k[E_2] + 2ak[E_2A] + \beta k[A EA]$$

Substituting for $[E_2]$, etc. in terms of $[E_0]$, we obtain:

$$\frac{-d[E]}{[E_0]} = k_{app} dt$$

and

$$\ln \frac{[E]_0}{[E]} = k_{app} t$$

where:

$$k_{app} = \frac{k(1 + \beta[A])}{K_A} \left(1 + \frac{[A]}{K_A}\right)$$

The $k_{app}$ for loss of activity will be numerically equal to twice the $k_{app}$ for dimer dissociation (one dissociation event loses two active sites). While $k_{app}$ is a complex function of $[A]$, the semi-log plots are linear at each $[A]$ regardless of the values of $\alpha$ and $\beta$. If $ak$ (the rate constant for the dissociation of singly occupied dimers) is the average of $k$ and $\beta k$, i.e. $\alpha = (1 + \beta)/2$, $k_{app}$ reduces to:

$$k_{app} = \frac{k(1 + \beta[A])}{K_A} \left(1 + \frac{[A]}{K_A}\right)$$

Equation 11 is identical to Equation 4 which was derived without regard to the oligomeric state of the enzyme.
Substituting for \([E]\) and \([EA]\) in Equation 12 we obtain:

\[
-\frac{d[E]}{[E]^2} = k_{\text{app}} \, dt
\]  

(15)

Equation 15 integrates to the usual second-order equation:

\[
\frac{1}{[E]} = k_{\text{app}} \, t + \frac{1}{[E]_0}
\]  

(16)

where:

\[
k_{\text{app}} = \frac{k}{\left(1 + \frac{[A]}{K_A}\right)^2 + \frac{2\alpha k}{K_A} \left(1 + \frac{K_a}{[A]}\right) \left(1 + \frac{K_a}{[A]}\right)^2}
\]  

(17)

or

\[
k_{\text{app}} = \frac{k \left(1 + \frac{2\epsilon[A]}{K_A} + \frac{\beta[A]^2}{K_A^2}\right)}{\left(1 + \frac{[A]}{K_A}\right)^2}
\]  

(18)

In agreement with Scheme III, \(k_{\text{app}}\) has limits of \(k\) at \([A] = 0\) and \(\beta k\) when \([A]\) is saturating. The expression for \(k_{\text{app}}\) cannot be simplified, but if \(\alpha = (\beta + 1)/2\) (i.e. \(\alpha\) is the average of \(k\) and \(\beta k\)), \(k_{\text{app}}\) reduces to:

\[
k_{\text{app}} = \frac{k \left(1 + \frac{\epsilon[A]}{K_A}\right)}{\left(1 + \frac{[A]}{K_A}\right)}
\]  

(19)

Equation 19 shows that \(k_{\text{app}}\) is a hyperbolic function of \([A]\). Thus, a replot of \(1/\Delta k\) versus \(1/[A]\) allows both \(\beta\) and \(K_A\) to be determined. \((\Delta k)\) is the difference between \(k_{\text{app}}\) at any \([A]\) and \(k\) when \([A] = 0\). Under certain limiting conditions \(k_{\text{app}}\) may appear to be a linear function of \([A]\) (see later, Equation 28).

If only \(EA\) dimerizes, \(k_{\text{app}}\) is given by Equation 20 (where \(\beta k\), the only relevant rate constant, is indicated simply as \(k\)):

\[
k_{\text{app}} = \frac{k}{\left(1 + \frac{K_a}{[A]}\right)^3}
\]  

(20)

\(K_a\) can be calculated from the ratio of \(k_{\text{app}}\) at any two \(A\) concentrations, \([A]_0\) and \([A]_1\). If \(k_{\text{app0}}/k_{\text{app1}} = n\), a final equation of the form \(aK_a^2 + bK_a + c = 0\) can be obtained where:

\[
a = \frac{n[A]_1 - [A]_0}{[A]_0^2 [A]_1^2}
\]  

(21)

\[
b = \frac{2(n[A]_0 - [A]_1)}{[A]_0 [A]_1}
\]  

(22)

\[
c = (n - 1)
\]  

(23)

With \(K_a\) known, \(k\) can be calculated from any \(k_{\text{app}}\). A plot of log \(k_{\text{app}}\) versus \(pA\) (e.g. \(pH\) if \(A = H^+\)) falls from a maximum of log \(k\) approaching a limiting slope of \(-2.0\) at high \(pA\). When \(k_{\text{app}} = 0.25 \, k\), \(pA = pK_A\).

A similar treatment can be applied to the situation where only \(E\) dimerizes. In this case, \(k_{\text{app}}\) is given by:

\[
k_{\text{app}} = \frac{k}{\left(1 + \frac{[A]}{K_A}\right)^3}
\]  

(24)

The ligand \(A\) is, in effect, a reversibly-bound inhibitor of the reaction. A plot of log \(k_{\text{app}}\) versus \(pA\) (e.g. \(pH\) if \(A = H^+\)) rises to a maximum of log \(k\). The limiting slope at very low \(pA\) is 2.0; \(pA = pK_A\) when \(k_{\text{app}} = 0.25 \, k\).

Effect of a Reversibly Bound Ligand on the Reassociation of Two Dissimilar Subunits—Suppose that an active heterodimer has been dissociated into its inactive component subunits which reassociate upon altering the solution conditions (\(pH\), temperature). Reassociation is a bimolecular and second-order process where at any time \([E]\), the concentration of one subunit (e.g. the catalytic subunit) equals \([E_*]\), the concentration of the other subunit (e.g. the regulatory subunit). A ligand (e.g. a substrate or effector) may affect the rate of reassociation by binding to one type of subunit and promoting a conformational change which favors or discourages interaction with the other type of subunit. While the situation sounds complex, it is actually simpler to analyze than the ligand-affected association of a homodimer. The reactions are shown below assuming that the ligand binds only to \(E_*\).

\[
E_a + E_* \xrightarrow{k} E_aE_*
\]

\[
E_a + E_* \xrightarrow{\beta k} E_aE_*
\]

Scheme IV

The rate of \(E_a\) disappearance (which equals the rate at which catalytic activity reappears) is given by:

\[
-\frac{d[E]}{dt} = [kK_a][E_a] + \beta k[E_a][E_*]
\]  

(25)

where \([E]\), the concentration of inactive type a subunits, is given by:

\[
[E]\ = [E_a] + [E_aA]
\]

Also, because the mixture of subunits was obtained by dissociating the heterodimer (or by adding equimolar concentrations of \(E_a\) and \(E_*\)), at any time:

\[
[E_*] = [E_a] + [E_aA]
\]

Substituting for \([E_*]\) \([E_aA]\), and \([E_*]\) in terms of \([E]\), we obtain:

\[
-\frac{d[E]}{[E]^2} = k_{\text{app}} \, dt
\]

where:

\[
k_{\text{app}} = \frac{k}{\left(1 + \frac{[A]}{K_A}\right)^3}
\]  

(26)

or

\[
k_{\text{app}} = \frac{k \left(1 + \frac{\epsilon[A]}{K_a}\right)}{\left(1 + \frac{[A]}{K_A}\right)^2}
\]  

(27)

A replot of \(1/\Delta k\) versus \(1/[A]\) will yield \(K_a\) and \(\beta\).

If \(\beta \gg 1\) and the \(A\) concentration range is \(\ll K_A\), Equation
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27 and Equation 19 approximate a linear function:

\[ k_{\text{app}} = k \left( 1 + \frac{\beta[A]}{K_A} \right) \quad \text{or} \quad k_{\text{app}} = \frac{\beta k}{K_A} [A] + k \quad \text{(28)} \]

Theoretically, the replot of \( k_{\text{app}} \) versus \([A]\) has intercepts of \( k\) (on the vertical axis) and \(-K_A/\beta\) (on the horizontal axis). In practice, the replot is likely to intersect the axes too close to the origin to provide reliable values.

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