Effect of Adenosine Triphosphate on Beef Cardiac Actomyosin*

A. L. Jacobson,† W. G. Cambell, and J. M. Adams

ABSTRACT: The velocity profiles for the calcium and magnesium activated ATP hydrolysis by beef cardiac actomyosin have been measured. In both cases the kinetics are complex, and a plateau region has been observed in the velocity profile. The data are consistent with either a modified single site London and Steck Model III or the multisited Teipel and Koshland model for enzyme systems. The rate and extent of superprecipitation in the presence of calcium or magnesium have also been measured for beef cardiac actomyosin. The velocity profiles for superprecipitation are sigmoidal. The differences in the type of curves obtained for superprecipitation and for ATP hydrolysis indicate that there may be a difference in the binding site (or sites) for hydrolysis and for contraction.

For many enzymes, detailed analysis of substrate–rate curves have provided information on the mechanism of substrate interaction with the enzyme. Various kinetic models have been proposed for complex enzyme kinetics. Cleland (1963) proposed a model in which the reactant (ATP) and the modifier (magnesium ions) interact to produce the active substrate (the Mg–ATP complex). London and Steck (1969) extended this concept and described a kinetic model in which a (metal) modifier and a reactant combine with the enzyme and each other. The modifier's dual role can produce a variety of complex kinetic curves including a peak velocity and/or a sigmoidal velocity curve. Teipel and Koshland (1969) described a model in which multiple binding sites would produce an intermediary plateau region in an enzyme saturation curve.

With actomyosin additional information concerning substrate–enzyme interactions can be obtained by measurement of the effect of substrate on the contractibility of the protein. This has been termed "superprecipitation" and has been measured by the turbidity changes in the actomyosin gel induced by ATP (Ebashi, 1961). Information concerning the number and function of ATP sites in various types of actomyosin has been obtained by comparison of the kinetics of hydrolysis and the kinetics of superprecipitation.

For rabbit skeletal actomyosin, measurements of binding constants have suggested the existence of two ATP sites per myosin oligomer. Tonomura and Morita (1959) measured the binding constants for pyrophosphate, and Kubo *et al.* (1965) measured the binding of trinitrobenzenesulfonate to rabbit myosin. The latter work suggested that the sites are not structurally equivalent. Levy and Fleisher (1965b) suggested that there is one ATP site for ATP hydrolysis and a separate second ATP site is necessary for contraction of the rabbit skeletal actomyosin. Levy and Ryan (1966) have shown that superprecipitation required the concerted action of two or more molecules of ATP, and, consequently, the velocity curves for superprecipitation are complex. Eisenberg and Moos (1968) proposed an alternate explanation to the assumption of separate hydrolytic and dissociation sites for ATP in actomyosin. They believe that the dissociating effect of ATP on actomyosin may be explained more simply by assuming the binding of ATP at the hydrolytic site may decrease the affinity for actin but does not completely prevent the actin binding. Komitz (1970) has found that ATPase activity and turbidity measurements on myofibrils and actomyosin from rabbit psoas muscle to be in general accord with the London–Steck (1969) Model III of both a basal- and a metal-activated state. Komitz's results also support the conclusions of Levy and Fleisher (1965a) that two or more molecules of ATP interacting at distinct sites are involved in superprecipitation.

In a series of papers Chaplain has investigated the kinetic behavior of insect actomyosin. The rate data for the hydrolysis of ATP by insect actomyosin does not fit a Michaelian hyperbola except at high calcium concentrations (Chaplain, 1967). Three substrate binding sites per actomyosin oligomer were suggested. An allosteric model for the action of actin on myosin has been proposed (Chaplain, 1966), and the existence of two or three possible states of the protein suggested (Chaplain, 1969). A further modification of this approach has been the postulation that the actomyosin complex exists in both enzymatically active and inactive states and that a more appropriate model for the kinetic behavior would be of two allosteric systems interacting with one another (Chaplain, 1969).

While there are basic similarities in the molecular and physiochemical properties of cardiac and skeletal myosin (McCarl *et al.*, 1969), there have been several observations that the cardiac actomyosin–ATP and the skeletal actomyosin–ATP systems differ in some of their properties. The ATPase activity and the "ATP sensitivity" of cardiac actomyosin were found by Benson *et al.* (1955) to decrease more rapidly with aging than with skeletal actomyosin. Finkel and Gergely (1961) found that the inhibition of cardiac myofibrillar ATPase by excess ATP was less marked than for the skeletal myofibrillar ATPase. Bárány *et al.* (1964) found a lower specific ATPase activity for rabbit cardiac actomyosin compared to rabbit skeletal actomyosin. Addition of skeletal F-actin to cardiac myosin and of cardiac F-actin to skeletal myosin produced actomyosins with properties almost equal to cardiac and skeletal actomyosin, respectively. These conclusions were also reached by Katz *et al.* (1966). Tada (1967) found that canine cardiac actomyosin at low ionic strength was not inhibited to the same extent by excess ATP as skeletal actomyosin and that the superprecipitation and ATPase activity of canine cardiac actomyosin became desensitized to calcium ions and EGTA on aging of the preparations much more rapidly than for skeletal actomyosin. Tada (1967) found also that at low substrate concentrations the canine actomyosin

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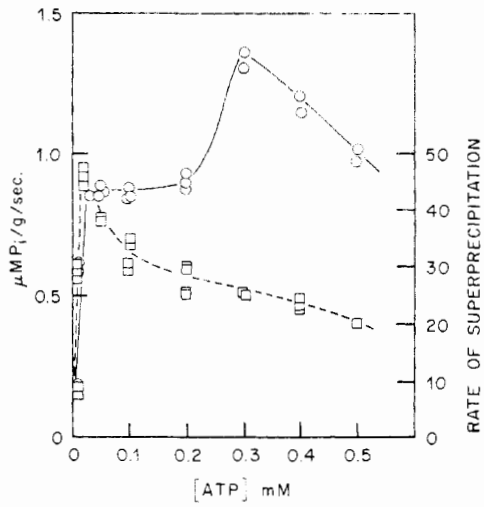

FIGURE 1: Hydrolysis of ATP and rate of superprecipitation as a function of ATP concentration. Solutions contained 0.06 mg/ml of protein, 0.03 M KCl, 1.0 mM $MgCl_2$, and 0.05 M Tris at pH 7.4: (○) ATP hydrolysis, μmoles of P_i/g per sec; (□) rate of superprecipitation, ΔOD/mg per ml per min.

ATPase activity velocity followed Michaelis–Menten kinetics until a critical concentration of ATP was reached above which the velocity decreased with increase of ATP concentrations. The ATPase activity kinetics for canine cardiac actomyosin appear to be of a simpler type than the kinetics of either rabbit psoas (Komitz, 1970) or of insect actomyosin (Chaplain, 1967). Kaldor *et al.* (1969) have shown that the extent of superprecipitation is smaller for cardiac actomyosin compared to skeletal actomyosin, and that the cardiac actomyosin is inhibited by lower KCl and [MgATP] concentrations.

In this work the kinetics of the calcium- and magnesium-activated hydrolysis of ATP by beef cardiac actomyosin has been studied. Since there have been reports of both Michaelis–Menten kinetics and more complex kinetics with actomyosin from other species, the possibility of complex kinetic behavior for beef cardiac actomyosin has been investigated. As suggested by Teipel and Koshland (1969) a number of closely spaced points have been measured to determine the possible applicability of the various kinetic models for a complex enzyme system. The type of kinetic behavior obtained for beef cardiac actomyosin has been compared to that previously obtained by other workers for insect and skeletal actomyosin as well as for cardiac actomyosin obtained from other species.

The rate of superprecipitation of beef cardiac actomyosin has also been determined. Comparisons have been made between the rate of superprecipitation and the rate of ATP hydrolysis to provide information concerning the number and function of ATP sites in beef cardiac actomyosin. With both rabbit skeletal and insect actomyosin this approach has suggested different ATP binding sites for ATP hydrolysis and contraction. This possibility has been considered here for beef cardiac actomyosin.

Materials and Methods

Double-distilled water was used throughout. All salts were of reagent grade. Extracting solutions were treated with Chelex 100 resin to remove divalent ions. The concentration of divalent ions in all solutions was checked by atomic absorption measurements. ATP was obtained from the Sigma Chemical Co.

TABLE I

ATP (mM)	μmoles of P_i/g per sec, Unwashed	μmoles of P_i/g per sec, Extensively Washed	% Activation
0.4	2.11	2.00	−5
0.5	2.22	2.52	+13
0.7	2.37	2.86	+21
1.0	2.65	2.88	+12

Cardiac actomyosin was prepared by the method of Stowring *et al.* (1966). Except where specified the precipitated protein was washed extensively to remove soluble protein (Schaub *et al.*, 1967). Protein solutions were stored at 2° at pH 7 in 0.6 M KCl. Protein concentration was measured by the micro-Kjeldahl technique. The factor 6.25 was used to convert per cent N to protein concentration.

The ATPase activity was measured at 25°, pH 7.4, in the presence of 0.05 M Tris buffer. The production of inorganic phosphate was measured by the method of Lecocq and Inesi (1966). All solutions were incubated for 10 min at 25° before aliquots of ATP were added. Aliquots (6–8) were taken at 100-sec intervals.

Superprecipitation was measured by a slight modification of the method used by Stowring *et al.* (1966), by following the changes in turbidity on addition of ATP at 500 mμ with a Zeiss spectrophotometer with a stirred, temperature-jacketed cell at 25° at pH 7.4. Greater reproducibility was obtained by precipitation of the actomyosin solution within the spectrophotometric cell rather than transfer of an aliquot of precipitated actomyosin to the cell. An aliquot of actomyosin solution was precipitated into a measured amount of stirred buffer solution which contained the appropriate concentration of magnesium or calcium within the cell. When the solution in the cell was stirred during protein addition, further changes in stirring speed did not affect either the rate or extent of superprecipitation.

Results

As shown in Table I, extensive washing of the precipitated protein increased the calcium-activated ATPase activity of cardiac actomyosin. The maximum increase was 21%. This is considerably less than the 40% increase for skeletal actomyosin reported by Schaub *et al.* (1967). The smaller change in cardiac actomyosin ATPase activity may be due, at least in part, to differences in experimental conditions. However, different binding constants for the soluble proteins to cardiac and skeletal actomyosin may also be responsible for the difference in activation by washing. Since some activation was observed with the cardiac actomyosin after extensive washing, the actomyosin used in the superprecipitation and the ATPase activity experiments was extensively washed.

The magnesium activated ATPase activity in the presence of 0.03 M KCl is shown in Figure 1. Excess magnesium (1 mM compared to maximum ATP concentration of 0.5 mM) is always present. The kinetic curve for the hydrolysis of ATP by precipitated actomyosin in the presence of excess magnesium ions is complex. A plateau region in the lower ATP concentration range (<0.2 mM ATP) is observed, and the maximum

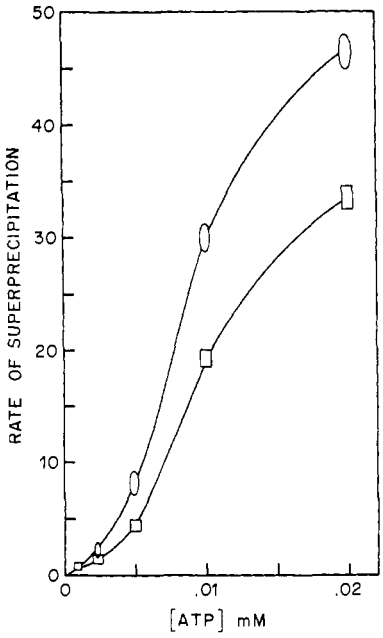

FIGURE 2: Rate of superprecipitation (ΔOD/mg per ml per min) as a function of ATP concentration. Solutions contained 0.06 mg/ml of protein, 0.03 M KCl, and 0.05 M Tris at pH 7.4. Standard deviation is indicated by size of the symbol: (○) 1.0 mM $MgCl_2$, average of three independent experiments; (□) 0.1 mM $MgCl_2$, average of two independent experiments.

experimentally measured velocity is reached at 0.3 mM ATP. The rate of superprecipitation under identical experimental conditions is also shown in Figure 1. The maximum rate of superprecipitation is reached at 0.02 mM ATP, and at higher ATP concentrations the rate of superprecipitation decreases. The rate curves for ATP hydrolysis and for superprecipitation are very different in character.

The initial part of the substrate rate curve for superprecipitation in 0.03 M KCl is shown in Figure 2. Both rate curves show the usual sigmoidal characteristics associated with an allosteric effect of the substrate on the enzyme. The extent of superprecipitation curve is shown in Figure 3. The sigmoidal behavior is more noticeable in the measurement of the rate of superprecipitation. The rate of superprecipitation also appears to be more sensitive to the concentration of magnesium ions

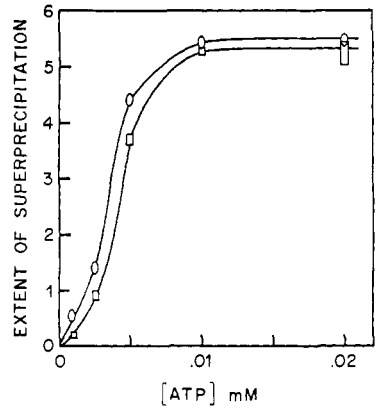

FIGURE 3: Extent of superprecipitation (ΔOD/mg per ml) as a function of ATP concentration. Symbols and conditions similar to Figure 2.

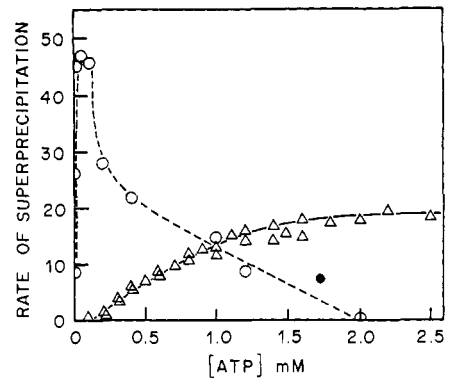

FIGURE 4: Rate of superprecipitation (ΔOD/mg per ml per min) as a function of ATP concentration. Solutions contained 0.1 mg/ml of protein, 0.03 M KCl, and 0.05 M Tris at pH 7.4: (○) 1.0 mM $MgCl_2$; (△) 10.0 mM $CaCl_2$.

present, and much larger changes in rate are observed in increasing from 0.1 to 1.0 mM $MgCl_2$ than in the measurement of extent of superprecipitation.

In Figure 4, a comparison is shown between the rates of superprecipitation at 0.03 M KCl in the presence of 1.0 mM $MgCl_2$ and 10.0 mM $CaCl_2$. The experimental data for $MgCl_2$ is for a different protein preparation than shown in Figure 1. Both the general behavior and the magnitude of the rate of superprecipitation do not appear to be dependent on the protein preparation. Replacement of the magnesium with calcium causes a large displacement of the rate of superprecipitation curves as a function of ATP concentration. The rate curve for superprecipitation in the presence of calcium appears sigmoidal.

The calcium-activated ATPase activity of beef cardiac actomyosin has also been measured with the protein in solution rather than in the precipitated form. The ATPase activity at 0.24 M KCl in the presence of 10 mM $CaCl_2$ is shown in Figure 5. A plateau region is also observed in this velocity curve before the maximum experimental velocity is reached. On storage of the protein solution (at 0.6 M KCl, 2°, pH 6.8),

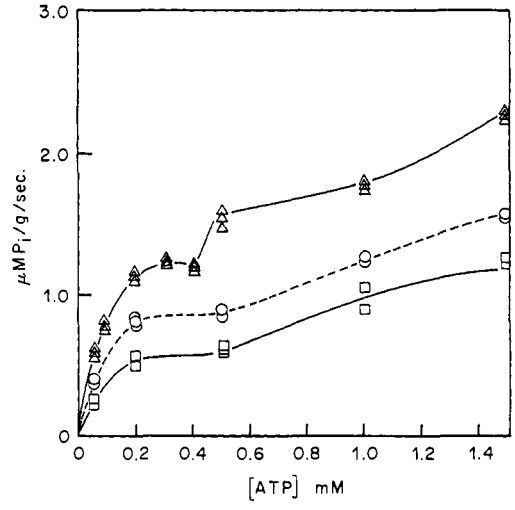

FIGURE 5: Hydrolysis of ATP as a function of ATP concentration. Solutions contained 0.12 mg/ml of protein, 0.24 M KCl, 10.0 mM $CaCl_2$, and 0.05 M Tris at pH 7.4. Freshly prepared actomyosin (△) and the same stock solution of actomyosin stored 3 days (○) and 7 days (□) are shown.

the relative activity decreases, but the plateau behavior is not affected by aging of the protein solution. The general shape of the velocity curve for actomyosin in solution is the same as in the precipitated form shown in Figure 1.

Discussion

The sigmoidal behavior of the kinetics of rate of superprecipitation suggests that an allosteric effect is operative. The superprecipitation rate curves for cardiac actomyosin resemble the superprecipitation curves obtained for insect actomyosin (Chaplain, 1966, 1969) and for rabbit skeletal actomyosin (Levy and Fleisher, 1965b). The displacement of the rate of superprecipitation curves to higher ATP concentrations by substitution of calcium for magnesium is similar to the behavior previously reported by Watanabe and Yasui (1965) for the effect of divalent ions on extent of superprecipitation.

Differences observed between superprecipitation and ATP hydrolysis kinetics were used by Levy and Fleisher (1965b) as an indication that contraction required reaction of ATP at two sites while hydrolysis occurred at only one of these sites. For beef cardiac actomyosin the maximum experimentally observed velocity occurs at a much higher ATP concentration for ATP hydrolysis than for superprecipitation. It appears that there are different sites operative for ATP hydrolysis and superprecipitation in this ATP concentration range. In the range of ATP concentrations (<0.1 mM) studied by Levy and Fleisher (1965b) the ATP hydrolysis for rabbit skeletal actomyosin followed simple kinetic behavior. In this low ATP concentration range the beef cardiac actomyosin also showed simple kinetic behavior and the deviations from Michaelis–Menten kinetics were only apparent at higher ATP concentrations.

An explanation of the kinetic behavior of the actomyosin ATPase base on the assumption that a mixture of enzymes is present appears reasonable since actomyosin is a complex protein. Myosin is expected to be present in any actomyosin preparation due to the dissociation of the actomyosin complex as well as by coprecipitation with the actomyosin. Hence, it is possible that a myosin type ATPase site is present in the actomyosin. However, while actomyosin is activated by magnesium ions at low ionic strength, myosin itself is inhibited by magnesium ions at low ionic strength. Sugden and Nehei (1969) have reported that the relative activity of myosin decreased as magnesium was added, and in the presence of 1 mM magnesium ions the relative activity of their myosin was zero with both 0.1 and 1.0 mM ATP addition. Their experimental conditions (0.05 M KCl, 0.33 mg/ml of protein, pH 7.4, 25°) are very similar to the experimental conditions employed with the beef cardiac actomyosin (0.03 M KCl, 0.1 mg/ml of protein, pH 7.4, 25°). Hence, under these experimental conditions myosin ATPase activity is inhibited, and it is unlikely that the plateau behavior of the actomyosin could be attributed to a myosin-type site in the presence of excess magnesium ions.

A further possibility for explanation of the plateau could be an assumption that the actomyosin exists in an equilibrium between monomeric and aggregated forms, and that the nature of the ATP site (or sites) depends on the aggregation. This possibility cannot be ruled out. However, the continued presence of the plateau behavior on aging of the actomyosin solutions makes this a less attractive possibility since some changes in aggregation would be expected on storage of the protein solution.

London and Steck (1969) have provided a model for enzyme kinetics based on a basal- and a metal-activated state. The authors have commented on the phenomenon of the velocity profile of an enzyme being initially concave, then sigmoid before the peak velocity is reached with a single site enzyme and their Model III. London and Steck believe that this phenomenon implies multiple paths of product formation. Komitz (1970) has used a slight modification of the London–Steck Model III as a model for the behavior of rabbit psoas actomyosin. Komitz assumed that the substrate was the [MgATP] complex and that there were three pathways to product. The velocity profile of beef cardiac actomyosin could also be of this type.

The rate analysis presented by Teipel and Koshland (1969) could also be used to simulate the kinetic behavior of beef cardiac actomyosin. The plateau behavior only occurs if there are more than two substrate binding sites, and if the relative magnitude of the intrinsic binding or catalytic constants first decreases, then increases as the enzyme is saturated. Both conditions are possible for the beef cardiac actomyosin. Two or more ATP binding sites have been proposed by Tonomura and Morita (1959), Kubo *et al.* (1965), and Levy and Fleisher (1965a,b) for rabbit skeletal actomyosin. Hence a kinetic model which assumes more than two ATPase sites for beef cardiac actomyosin is not unreasonable. Teipel and Koshland (1969) attribute the possible cause of decreased affinity for substrate to either a ligand-induced conformational change, or to a mixture of two or more proteins containing subunits each with a different intrinsic binding constant. Both explanations appear feasible for actomyosin. The increased affinity for substrate was attributed to a positive cooperative action of the allosteric type. An allosteric effect has been shown to exist in insect actomyosin (Chaplain, 1969), and such an effect appears feasible for beef cardiac actomyosin.

Either the single site London and Steck Model III or the multisite model of Teipel and Koshland could produce the plateau behavior reported here for beef cardiac actomyosin. As pointed out by Teipel and Koshland (1969) it is not possible to unequivocally establish the kinetic model for complex systems. The assumptions inherent in both models are consistent with the previously reported behavior of other types of actomyosin.

The kinetics for the hydrolysis of ATP by beef cardiac actomyosin appear to be more complex than the kinetic behavior reported for canine actomyosin (Tada, 1967). Teipel and Koshland (1969) have suggested that complex kinetics could be masked in enzyme systems if the experimental data do not contain a number of closely spaced points. It is possible that more complex kinetic characteristics may be revealed for the canine cardiac actomyosin with additional data. However it is also possible that the differences are due to species differences and beef cardiac actomyosin more closely resembles insect or rabbit psoas actomyosin in its kinetic behavior.

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Association–Dissociation and Abnormal Kinetics of Bovine α-Acetylgalactosaminidase*

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ABSTRACT: Gel filtration and kinetic studies show that bovine α-acetylgalactosaminidase exists in dilute solutions as a mixture of active oligomer(s) and inactive monomer. The rapidly reversible equilibrium between these species is temperature dependent and is linked with binding of substrate or competitive inhibitor. Association is enhanced by increased enzyme concentration, increased substrate concentration, or decreased temperature.

The most associated species has a molecular weight of about 155,000 and the monomer has a molecular weight between 30,000 and 42,000. These findings account for a previously observed kinetic abnormality, the decrease of apparent specific activity on dilution, now shown to apply also to the corresponding enzymes of the rabbit, turtle, and frog, but not those of the pig, rat, guinea pig, chicken, fish, or earthworm.

Unusual kinetic behavior was found earlier for a partially purified preparation of α-acetylgalactosaminidase from beef liver, using phenyl *N*-acetyl-α-galactosaminide as a test substrate (Weissmann and Hinrichsen, 1969). The phenomena observed included a decline in apparent specific activity of the enzyme on dilution. This abnormality was most prominent at low substrate concentrations and higher temperatures. The rapidly reversible dissociation of an active enzyme into inactive subunits was offered as a possible, but unproven, explanation.

Parallel physical and kinetic observations now demonstrate such a facile association–dissociation, which is linked with binding of substrate or competitive inhibitor. This enzyme accordingly affords an illustration of the interaction of "ligand" binding[1] with protein polymerization (Klapper and Klotz, 1968; Frieden, 1967; Nichol *et al.*, 1967) apparently simpler than most of the numerous well documented cases presently known, which generally involve interaction of complex regulatory enzymes with allosteric effectors (Frieden and Colman, 1967; Vagelos *et al.*, 1963; Numa *et al.*, 1967; Datta *et al.*, 1964; Hirata *et al.*, 1965; Scrutton and Utter, 1965; Iwatsuki and Okazaki, 1967; Maley and Maley, 1968; LeJohn *et al.*, 1969; DeVincenzi and Hedrick, 1970; Constantinides and Deal, 1970; Leary and Kohlaw, 1970; Long *et al.*, 1970).

Experimental Section

Materials. The beef liver α-acetylgalactosaminidase preparation whose kinetic and chromatographic behavior form the subject of this work was the partially purified specimen described earlier (Weissmann and Hinrichsen, 1969). Its activity had not changed significantly during storage for

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[1] For present purposes, "ligand" has reference only to binding of small molecules and disregards protein–protein binding, which is referred to explicitly.