Structure of the Oxalate–ATP Complex with Pyruvate Kinase: ATP as a Bridging Ligand for the Two Divalent Cations†

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ABSTRACT: The 2 equiv of divalent cation that are required cofactors for pyruvate kinase reside in sites of different affinities for different species of cation [Baek, Y. H., & Nowak, T. (1982) *Arch. Biochem. Biophys. 217*, 491–497]. The intrinsic selectivity of the protein-based site for Mn(II) and of the nucleotide-based site for Mg(II) has been exploited in electron paramagnetic resonance (EPR) investigations of ligands for Mn(II) at the protein-based site. Oxalate, a structural analogue of the enolate of pyruvate, has been used as a surrogate for the reactive form of pyruvate in complexes with enzyme, Mn(II), Mg(II), and ATP. Addition of Mg(II) to solutions of enzyme, Mn(II), ATP, and oxalate sharpens the EPR signals for the enzyme-bound Mn(II). Superhyperfine coupling between the unpaired electron spin of Mn(II) and the nuclear spin of ^{17}O, specifically incorporated into oxalate, shows that oxalate is bound at the active site as a bidentate chelate with Mn(II). Coordination of the γ-phosphate of ATP to this same Mn(II) center is revealed by observation of superhyperfine coupling from ^{17}O regiospecifically incorporated into the γ-phosphate group of ATP. By contrast, ^{17}O in the α-phosphate or in the β-phosphate groups of ATP does not influence the spectrum. Experiments in ^{17}O-enriched water show that there is also a single water ligand bound to the Mn(II). These data indicate that ATP bridges Mn(II) and Mg(II) at the active site. A close spacing of the two divalent cations is also evident from the occurrence of magnetic interactions for complexes in which 2 equiv of Mn(II) are present at the active site. The structure for the enzyme–Mn(II)–oxalate–Mg(II)–ATP complex suggests a scheme for the normal reverse reaction of pyruvate kinase in which the divalent cation at the protein-based site activates the keto acid substrate through chelation and promotes phospho transfer by simultaneous coordination to the enolate oxygen and to a pendant oxygen from the γ-phosphate of ATP.

The requirements of pyruvate kinase for activation by inorganic cations differ from those of many other enzymes that catalyze phospho-transfer reactions. In addition to a specific requirement for a monovalent cation (Boyer et al., 1942), activity of pyruvate kinase requires 2 equiv of divalent cation (Gupta et al., 1976; Gupta & Mildvan, 1977; Baek & Nowak, 1982). In the normal reaction, the inorganic cations together with the proton that adds to the enolate of pyruvate balance the net negative charges carried by the two substrates at neutral pH:

$$\text{phosphoenolpyruvate}(3-) + H^+ + ADP^{3-} \underset{}{\overset{2M^{2+},\,M^+}{\rightleftharpoons}} ATP^{4-} + \text{pyruvate}(1-)$$

The geometrical arrangement of the inorganic cofactors at the active site of pyruvate kinase and their possible roles in the reaction have been studied extensively (Nowak & Suelter, 1981). The enzyme binds 1 equiv of divalent cation per subunit in the absence of substrates (Reuben & Cohn, 1970). Results from X-ray crystallographic studies with the enzyme from cat muscle suggest that the carboxylate of Glu-271 and main-chain carbonyl groups from Ala-292 and Arg-293 may provide ligands for the metal ion at this site (protein-based site) (Muirhead et al., 1986). The second divalent cation likely resides at the active site as a complex with the nucleotide substrate (the nucleotide site) (Gupta et al., 1976; Dunaway-Mariano et al., 1979). NMR[1] relaxation measurements with Mn(II) and Co(II) as paramagnetic probes at the protein site and with the exchange-inert $Cr^{III}ATP$ complexes at the nucleotide site have established that all three inorganic cofactors and the two substrates bind in close proximity (Van Divender & Grisham, 1985; Mildvan et al., 1976). Although outer-sphere interactions between substrates and the divalent cation at the protein-based site have been inferred from NMR relaxation measurements (Mildvan et al., 1976), a more direct role for this cation in binding and activation of the substrates may have been missed because of chemical exchange limitations on nuclear spin relaxation rates (Cohn & Reed, 1982). In fact, earlier EPR studies showed that the coordination sphere of enzyme-bound Mn(II) was influenced significantly upon binding of pyruvate, P-enolpyruvate, and analogues of these substrates (Reed & Cohn, 1973).

Baek and Nowak (1982) have shown that additions of Mn(II) to assay mixtures containing an excess of Mg(II) stimulate activity of pyruvate kinase. The synergistic activation obtained with low concentrations of Mn(II) suggested that

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[1] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [α-^{17}O]ATP, [α,α-$^{17}O_2,\alpha\beta$-^{17}O]ATP; [β-^{17}O]ATP, [β,β-$^{17}O_2,\beta\gamma$-^{17}O]ATP; [γ-^{17}O]ATP, [γ,γ,γ-$^{17}O_3$]ATP; *D* and *E*, axial and rhombic zero-field splitting terms in the spin Hamiltonian for Mn(II); P-enolpyruvate, phosphoenolpyruvate.

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Mg(II) partitions preferentially to the nucleotide site whereas Mn(II) binds preferentially to the enzymic site. The differential affinities of the two sites for Mg(II) and Mn(II) may be exploited in spectroscopic investigations of "hybrid" complexes in which both sites for metal ions are occupied but in which Mn(II) resides virtually exclusively at the protein-based site. With a diamagnetic metal–nucleotide complex present at the active site, a condition of "magnetic dilution" prevails, and this permits use of EPR spectroscopy together with ^{17}O-labeled substrates to probe liganding interactions to Mn(II) with the full complement of cofactors present. Complexes of the enzyme with Mn(II) and the competitive inhibitor, oxalate, have favorable EPR properties (Reed & Morgan, 1974). This paper reports the results of EPR investigations of enzymic complexes in which Mn(II), oxalate, Mg(II), and ATP are present at the active site. Magnetic interactions that occur when two paramagnetic metal ions are present at the active site are also investigated. The experiments reveal a structure in which ATP functions as a bridging ligand for the two divalent cations and in which both oxalate and ATP (γ-phosphate) bind directly to the divalent cation at the protein-based site.

Experimental Procedures

Materials. Pyruvate kinase was isolated from rabbit muscle according to the method of Tietz and Ochoa (1958) with modifications as described by Ash et al. (1984). Specific activities of the preparations were in excess of 200 IU/mg in the coupled assay with lactate dehydrogenase at 21 °C. Prior to use in EPR experiments, solutions of the enzyme were subjected to chromatography on a column (2.5 × 90 cm) of Sephacryl S-200 with an equilibration and running buffer of 50 mM Hepes/KOH, pH 7.5, with 75 mM KCl. Solutions of enzyme were concentrated in this same buffer with a collodion bag apparatus. Water enriched in ^{17}O was obtained from Yeda Research and Development and from Monsanto.

Oxalate enriched to 54 ± 3 atom % in ^{17}O was prepared by incubation of oxalic acid in ^{17}O-enriched water (61 atom % ^{17}O) for 7 days (Ash, 1982). ^{17}O enrichment in the oxalate was determined by mass spectrometry. Samples of ATP with regiospecific incorporation of ^{17}O in the α-, β-, and γ-phosphate groups were prepared as described by Leyh et al. (1985). ^{17}O enrichment in the labeled ATP was determined by ^{31}P NMR at 145 MHz by the method of Tsai (1979).

EPR Measurements. EPR spectra were obtained at 35 GHz (Q-band) with Varian spectrometers equipped with a Varian E-110 microwave bridge and accessories. Spectral simulations were carried out as described previously (Reed & Markham, 1984; Moore & Reed, 1985). In the presence of oxalate the dissociation constant for Mn(II) from the enzyme–oxalate complex is 1 μM (Reed & Morgan, 1974). At the concentrations of enzyme and oxalate used in the experiments, there is a virtually stoichiometric binding of Mn(II) to the enzyme–oxalate complex. In the presence of ATP and Mg(II) there are additional competing equilibria between Mg(II) and Mn(II) for the various sites. The results of Baek and Nowak (1982) indicate that Mn(II) is able to compete effectively for the divalent cation site on the enzyme against a vast excess of Mg(II). The EPR data show (see Results and Discussion) that all of the EPR signals arise from Mn(II) bound to the enzyme under conditions of excess [enzyme], [ATP], and [Mg^{2+}]. Moreover, with ^{17}O labels in the ligands (oxalate, ATP, and H_2O) it is possible to use the EPR data for Mn(II) to determine where the Mn(II) is coordinated in enzymic complexes with both species of divalent metal ion bound.

Samples used for comparison of EPR spectra with labeled and unlabeled ligands were prepared from a common stock solution that contained all of the components except for the ligand of interest. The signal-to-noise ratio in the spectra was such that amplitudes of EPR signals for separate samples matched to within ±2–3%. Samples used for comparison of EPR spectra in labeled and unlabeled water were prepared from a common stock solution. Samples of equal volumes were lyophilized and redissolved either in ^{17}O-enriched water or in normal water.

Identification of Ligands. Superhyperfine coupling between the nuclear spin of ^{17}O ($I = {}^5/_2$) and the unpaired electron spin of Mn(II) can be used to identify oxygen ligands for Mn(II) (Reed & Leyh, 1980). The magnitude of ^{17}O–Mn(II) superhyperfine coupling is smaller than the intrinsic line widths of the EPR signals for Mn(II) in complexes with proteins, and the splitting[2] of the EPR signals due to coupling with ^{17}O is unresolved. The superhyperfine coupling does, however, contribute an inhomogeneous broadening to the EPR signals that is readily detectable whenever the line widths of the signals are comparable to the width of the sextet manifold resulting from the ^{17}O superhyperfine coupling (Reed & Leyh, 1980). Moreover, experiments with the two epimers of [α-^{17}O]ADP in complexes with creatine kinase (Leyh et al., 1982) have clearly demonstrated that, even with narrow EPR signals, the ^{17}O must be bound directly to Mn(II) to give a measureable inhomogeneous broadening in the EPR signals.

Determination of Number of Coordination Sites for a Ligand. Knowledge of the ^{17}O enrichment in a ligand permits one to determine the number of coordination sites for the ligand in the complex by a spectral subtraction procedure (Reed & Leyh, 1980). The spectrum that is observed for a sample containing an ^{17}O-enriched ligand is a superposition of spectra for complexes with Mn(II) coordinted to ^{17}O and to the nonmagnetic isotopes of oxygen. Those species with Mn(II) bound exclusively to nonmagnetic isotopes of oxygen (^{16}O and ^{18}O) contribute a spectrum that is identical in line shape with that of the control sample with unlabeled ligand whereas those complexes in which Mn(II) is bound to one or more ^{17}O ligands give broader signals due to the unresolved superhyperfine coupling. The statistical distribution of complexes with various combinations of magnetic and nonmagnetic isotopes of oxygen as ligands depends upon the ^{17}O enrichment in the ligand and on the number of sites for that ligand in the complex. The fraction F_n of the sample that has only nonmagnetic isotopes of oxygen as ligands is given by

$$F_n = [f(^{16}O) + f(^{18}O)]^n \quad (1)$$

where n is the number of coordination sites occupied by the ligand in question and $f(^{16}O) + f(^{18}O)$ is the fractional composition of nonmagnetic isotopes of oxygen in the labeled ligand. The analysis involves obtaining the difference spectrum:

$$L_d = L_e - F_n L_u \quad (2)$$

where L_d is the line shape of the difference spectrum and L_e and L_u are respectively the line shapes of spectra for the enriched and unenriched samples. An underestimate of n results in anomalous features in the line shapes of difference spectra that appear at positions where there are local maxima or

[2] The manifold of superhyperfine coupling from a single ^{17}O ligand is a sextet of transitions of equal intensity separated by the superhyperfine coupling constant. The manifold of superhyperfine coupling from two equivalent ^{17}O ligands is an 11-line pattern with a separation equal to the superhyperfine coupling constant and relative intensities of 1:2:3:4:5:6:5:4:3:2:1.

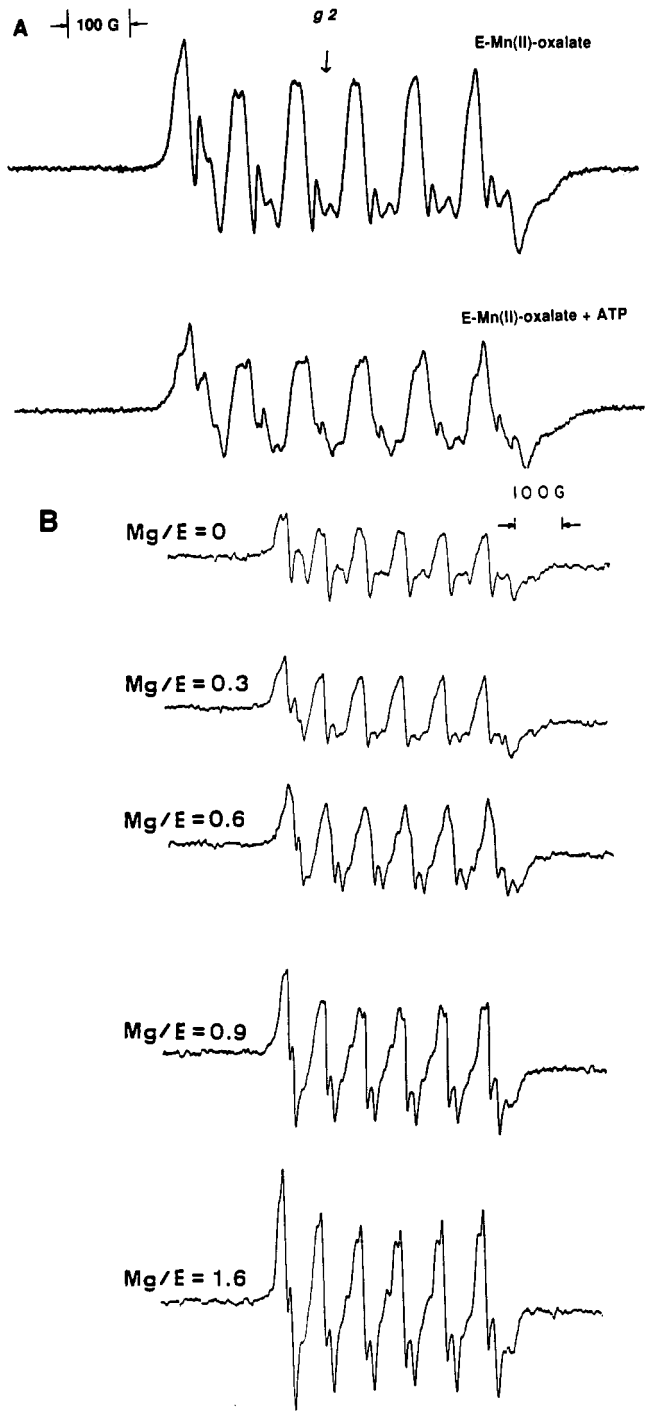

FIGURE 1: EPR spectra (Q-band) for solutions of pyruvate kinase–Mn(II)–oxalate without and with ATP and Mg(II) present. (A) Solutions contained 50 mM Hepes/KOH, pH 7.5, 75 mM KCl, 2.5 mM enzyme sites, 6.2 mM potassium oxalate, and 1.5 mM $MnCl_2$; addition of 2.4 mM ATP. (B) Solutions contained 50 mM Hepes/KOH, pH 7.5, 75 mM KCl, 3.5 mM enzyme sites, 7.9 mM potassium oxalate, 1.2 mM $MnCl_2$, and 2.8 mM ATP; additions of $MgCl_2$ as indicated. All spectra were obtained from the solution phase at 0 °C.

minima in the line shape of the spectrum for the unlabeled sample.

Results and Discussion

EPR Spectra for Complexes with Mg(II) and Mn(II). EPR spectra for a solution of enzyme, limiting [$MnCl_2$], and oxalate with and without ATP are shown in Figure 1A. The addition of ATP results in a decrease (~30%) in the amplitude of the EPR spectrum for the bound Mn(II). The decrease in

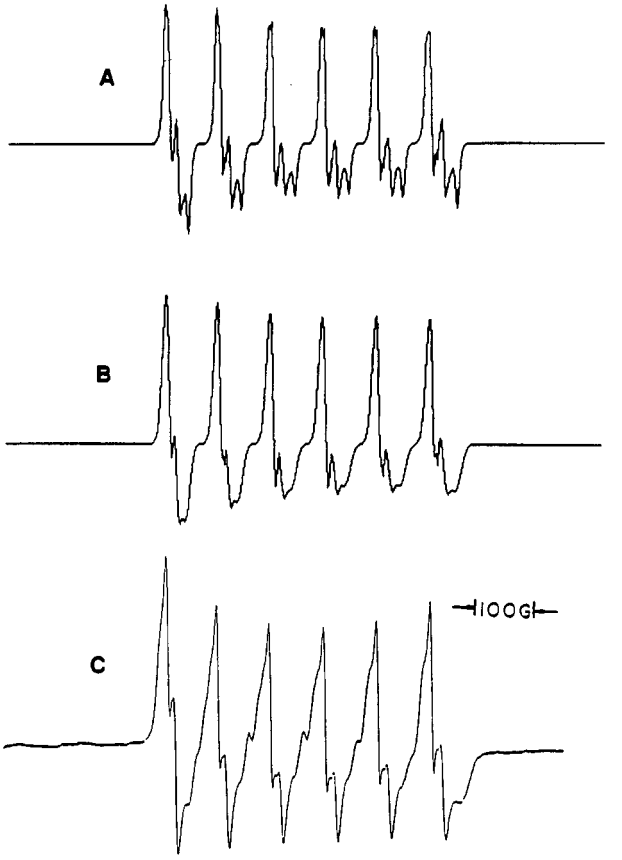

FIGURE 2: Simulated (A and B) and experimental (C) EPR spectra for the complex of pyruvate kinase–Mn(II)–oxalate–Mg(II)–ATP. (A) Parameters used in the simulation: D = 270 G, E = 45 G, $A(^{55}Mn)$ = 90 G, and Lorentzian line width parameter 4 G. (B) Same parameters used as in (A) with a Gaussian distribution in D with a half-width of 30 G (spectra for nine values of D were summed with amplitudes weighted as a Gaussian). (C) Experimental spectrum (for conditions see legend for Figure 3A).

the amplitudes of the signals is likely due to formation of new species such as enzyme–Mn(II)–oxalate–Mn(II)–ATP, which have broader signals (see below). A new set of signals grows into the spectrum upon additions of $MgCl_2$ to solutions of enzyme, Mn(II), oxalate, and ATP (Figure 1B). At concentrations of $MgCl_2$ sufficient to saturate[3] the metal nucleotide site on the enzyme, the spectrum for the enzyme-bound Mn(II) exhibits resolution superior to that of the ternary complex enzyme–Mn(II)–oxalate. Second-order fine structure in this $M_s = {}^1/_2 \leftrightarrow M_s = -{}^1/_2$ transition for Mn(II) shows that Mn(II) has not been displaced from the macromolecular complex by Mg(II). Positions of major features in the EPR spectrum for the enzyme–Mn(II)–oxalate–Mg(II)–ATP complex are reproduced in simulations with $D = 270 \pm 30$ G and $E/D = 0.16$ (see Figure 2). Simulation of the spectrum with a single set of parameters indicates that the EPR signals arise from a single dominant species of bound Mn(II).

In analogous experiments with ADP replacing ATP (data not shown) the spectrum characteristic of the ternary complex enzyme–Mn(II)–oxalate persists without changes in amplitude

[3] The observations indicate that there is a decided preference for formation of complexes with Mn(II) at one subsite and Mg(II) at the other subsite as indicated by the previous activity assays (Baek & Nowak, 1982). Other species such as enzyme–Mn(II)–oxalate–Mn(II)–ATP, enzyme–Mg(II)–oxalate–Mn(II)–ATP, and enzyme–Mg(II)–oxalate–Mg(II)–ATP are undoubtedly present at much lower concentrations. Experimental conditions for optimum expression of the EPR signals for the enzyme–Mn(II)–oxalate–Mg(II)–ATP species were obtained by varying the concentrations of ATP and Mg(II).

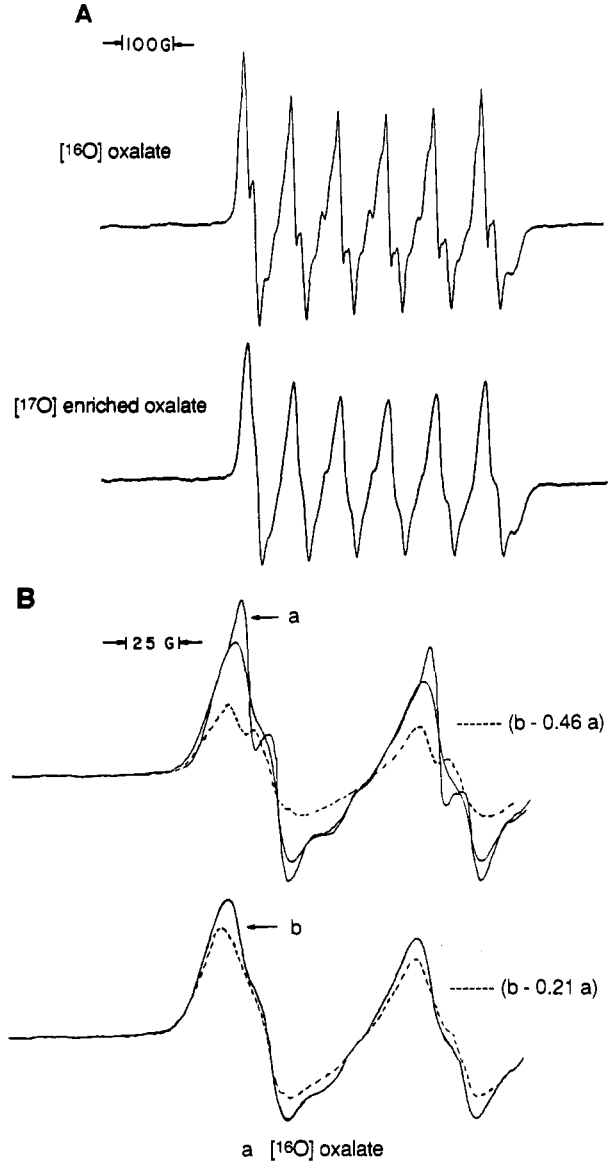

FIGURE 3: EPR spectra (Q-band) for solutions of pyruvate kinase–Mn(II)–oxalate–Mg(II)–ATP with ^{17}O-labeled and unlabeled oxalate. (A) Solutions contained Hepes/KOH, pH 7.5, 75 mM KCl, 3.4 mM enzyme sites, 1.4 mM $MnCl_2$, 2.9 mM ATP, 3.4 mM $MgCl_2$, and 6.3 mM unlabeled or labeled potassium oxalate. Spectra were obtained in the solution phase at −4 °C. (B) Expansions of the two lowest field ^{55}Mn hyperfine components. Dashed curves are difference spectra (representing the spectrum for the ^{17}O-liganded species) obtained for a monodentate [top, $F_n = 0.46$ (see eq 1)] or a bidentate (bottom, $F_n = 0.21$) coordination scheme for oxalate. The difference spectrum for the monodentate model exhibits anomalous features at positions corresponding to maxima or minima in the spectrum for the unlabeled sample. The appearance of these sharp features in the difference spectrum indicates that a smaller fraction of the sample exists with nonmagnetic isotopes of oxygen as ligands (i.e., oxalate is a bidentate ligand). The distribution of the Mn(II) species in the ^{17}O-enriched sample is 21% $^{17}O_0$, 50% $^{17}O_1$, and 29% $^{17}O_2$, where the subscript refers to the number of resident ^{17}O ligands. The dashed curve (bottom) is a superposition of spectra for the two ^{17}O-liganded species: 50% $^{17}O_1$ and 29% $^{17}O_2$.

or line shape, upon additions of $MgCl_2$ up to the concentration of active sites. This observation indicates that Mn(II), bound at the protein–oxalate subsite, does not experience any significant structural influences from binding of $Mg^{II}ADP$ at the metal–nucleotide subsite.

Measurements with ^{17}O-Enriched Oxalate. Experiments with ^{17}O-enriched oxalate were initiated in order to define the

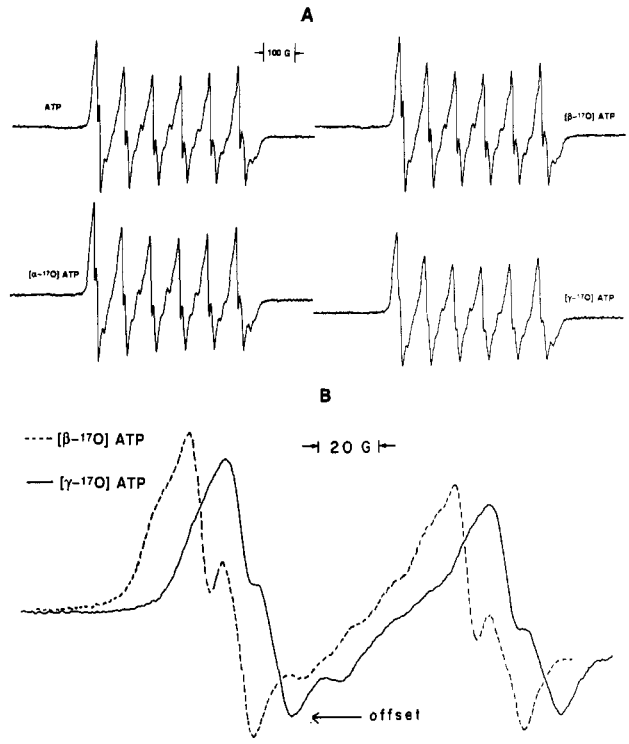

FIGURE 4: EPR spectra (Q-band) for solutions of pyruvate kinase–Mn(II)–oxalate–Mg(II)–ATP with unlabeled ATP and three forms of regiospecifically labeled ATP. Solutions contained Hepes/KOH, pH 7.5, 75 mM KCl, 2.5 mM enzyme sites, 1.2 mM $MnCl_2$, 5.1 mM potassium oxalate, 3.7 mM $MgCl_2$, and 2.4 mM ATP or labeled ATP. ^{17}O enrichment in the labeled forms of ATP was 41 ± 4%. Spectra were obtained at 1 °C. (B) Expansion of the two lowest field ^{55}Mn hyperfine components in spectra for samples with [β-^{17}O]- and [γ-^{17}O]ATP. Spectra in (B) are offset on the abscissa.

position occupied by Mn(II) in the complex with ATP and Mg(II) also present. EPR spectra for concentration-matched samples with oxalate and ^{17}O-enriched oxalate are shown in Figure 3A. EPR signals for Mn(II) in the sample with ^{17}O-enriched oxalate are inhomogeneously broadened by the superhyperfine interaction with ^{17}O. The number of ligands contributed to oxalate, n, can be determined by the spectral subtraction method (see Experimental Procedures) as shown in Figure 3B. The results of the spectral analysis show that oxalate is a bidentate ligand for the enzyme-bound Mn(II). Thus, the bidentate coordination between Mn(II) and oxalate in the ternary complex enzyme–Mn(II)–oxalate (Ash, 1982) remains intact upon binding of Mn(II)ATP at the active site.

Experiments with ^{17}O-Labeled ATP and Labeled Water. The possibility that the spectral effects observed upon binding of $Mg^{II}ATP$ to the enzyme–Mn(II)–oxalate complex might be due to a direct interaction of the ATP with the enzyme-bound Mn(II) was investigated in experiments with regiospecifically ^{17}O-labeled forms of ATP. EPR spectra for mixtures of enzyme, oxalate, Mn(II), Mg(II), and labeled forms of ATP are compared with that for unlabeled ATP in Figure 4. Signals in the EPR spectrum for the sample with γ-^{17}O-enriched ATP exhibit a uniform inhomogeneous broadening whereas spectra for α-^{17}O-enriched ATP and β-^{17}O-enriched ATP match that for the sample with unlabeled ATP. The fact that ^{17}O in the α-phosphate or in the β-phosphate of ATP is without an effect shows that the inhomogeneous broadening from ^{17}O in the γ-phosphate group is not due to a superposition of signals for a second species in which Mn(II) is bound at the nucleotide site. Furthermore, the same spectral features are broadened by the ^{17}O in the oxalate and in the γ-phosphate of ATP. These data demon-

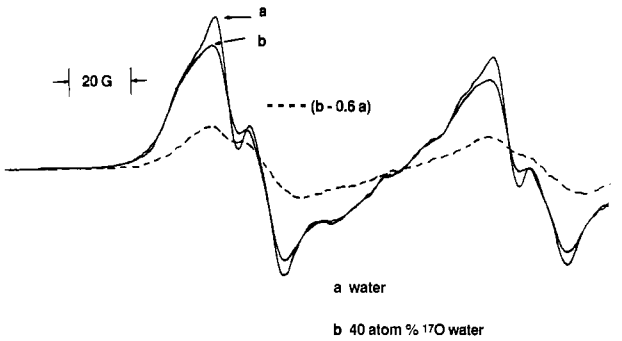

FIGURE 5: EPR spectra (Q-band) for solutions of pyruvate kinase–Mn(II)–oxalate–Mg(II)–ATP obtained in water and in water enriched (40 atom %) in ^{17}O. Solutions contained Hepes/KOH, pH 7.5, 75 mM KCl, 2.5 mM enzyme sites, 1.2 mM $MnCl_2$, 5.1 mM potassium oxalate, 3.7 mM $MgCl_2$, and 2.4 mM ATP. Spectra were obtained at 1 °C. The two lowest field ^{55}Mn hyperfine components are shown. The dashed curve is the difference spectrum corresponding to a single site [$n = 1$, $F_n = 0.6$ (see eq 1)] for water in the complex. In this case, there are no anomalies in the difference spectrum for $n = 1$.

strate that there is direct coordination between Mn(II) at the protein, oxalate-based site and the γ-phosphate of ATP.

The extent of ^{17}O-induced inhomogeneous broadening is greater in the spectrum obtained with ^{17}O-enriched oxalate than for that with γ-^{17}O-enriched ATP. This difference is, however, quantitatively consistent with the differences in ^{17}O enrichment of the oxalate (54 ± 2%) and ATP (41 ± 4%) and with the fact that oxalate is a bidentate ligand [i.e., in the sample of labeled oxalate 79% of the Mn(II) experiences superhyperfine coupling from ^{17}O, whereas with the sample of γ-^{17}O-enriched ATP only 40% of the Mn(II) has an ^{17}O ligand].

Signals for samples of the complex enzyme–Mn(II)–oxalate–Mg(II)–ATP prepared in $H_2{}^{17}O$ (40 atom % enrichment) exhibit inhomogeneous broadening (see Figure 5) that is quantitatively comparable to that observed in the signals for the sample with [γ-^{17}O]ATP with similar isotopic enrichment.[4] Analysis of the data for samples in labeled and unlabeled water indicates that there is a single site for water in the complex.

The EPR measurements with ^{17}O-labeled ligands reveal four ligands for Mn(II) at the protein-based site. Thus, two oxygens from oxalate and single oxygens, respectively, from the γ-phosphate of ATP and from a water molecule are bound to Mn(II). Although these EPR experiments do not define the exact position of Mg(II) in the complex, it is reasonable to surmise that Mg(II) is also coordinated to the ATP (Baek & Nowak, 1982) and that ATP bridges Mg(II) and Mn(II) at the active site.

Complexes with Two Paramagnetic Ions. The experiments described above indicate that the polyphosphate chain of ATP links the two divalent cations. The implied proximity of the two cations would likely result in a significant magnetic interaction whenever both sites are occupied by paramagnetic species. In a titration of a solution of enzyme, oxalate, and ATP with $MnCl_2$, one expects the enzyme–Mn(II)–oxalate site to fill initially [i.e., the protein, oxalate-based site has a higher affinity for Mn(II)] with population of the nucleotide site occurring at higher concentrations of Mn(II). EPR spectra corresponding to various points in such a titration are shown

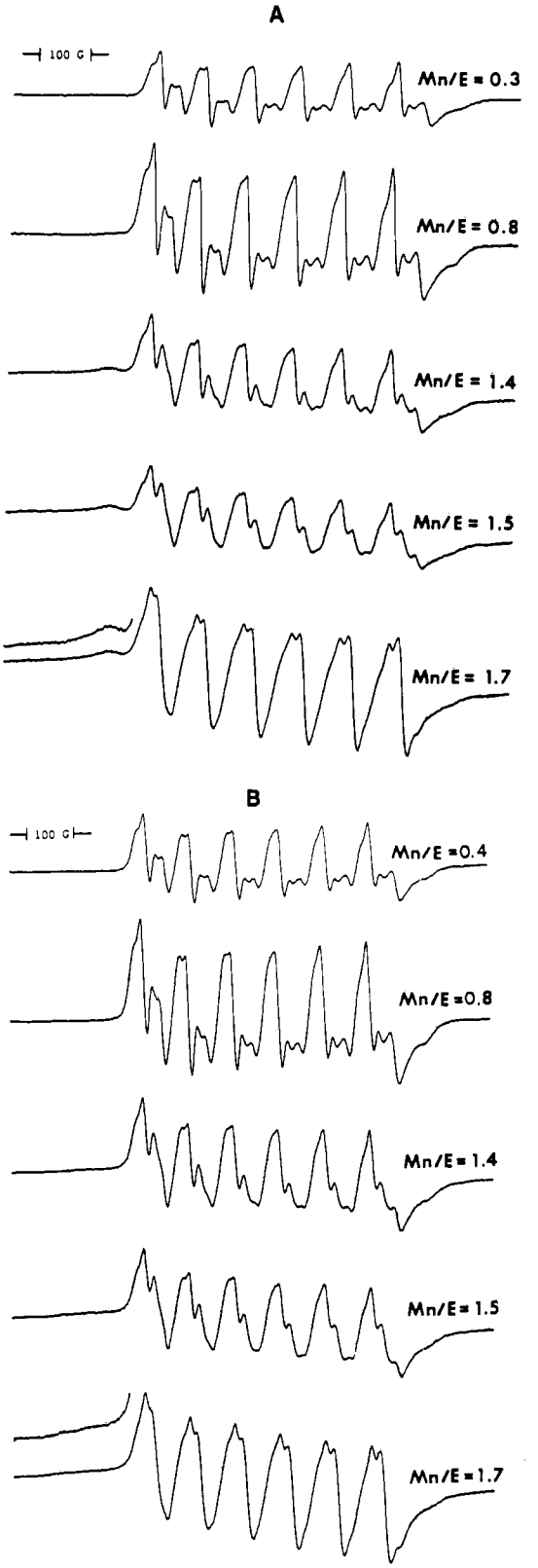

FIGURE 6: (A) EPR spectra (Q-band) for solutions of pyruvate kinase–oxalate–ATP with varying [Mn^{2+}]. Solutions contained Hepes/KOH, pH 7.5, 75 mM KCl, 4.5 mM enzyme sites, 6.5 mM potassium oxalate, and 3.1 mM ATP; $MnCl_2$ is as indicated. Spectra were obtained in the solution phase at −3 °C. The inset on the bottom spectrum was taken at a 2-fold higher gain setting. (B) EPR spectra (Q-band) for solutions of pyruvate kinase–oxalate–ADP with varying [Mn^{2+}]. Solutions contained Hepes/KOH, pH 7.5, 75 mM KCl, 4.2 mM enzyme sites, 5.4 mM potassium oxalate, and 3.9 mM ATP; $MnCl_2$ is as indicated. Spectra were obtained for the solution phase at −1 °C. The inset on the bottom spectrum was taken at a 2-fold higher gain setting.

[4] Inhomogeneous broadening in the spectra for samples with [γ-^{17}O]ATP (41% enrichment) or with 40% $H_2{}^{17}O$ reduces the amplitudes of the signals (relative to those for the unlabeled samples) for each sample by 18 ± 2%. Differences in the magnitudes of ^{17}O superhyperfine coupling constants for ^{17}O in a water ligand vs. ^{17}O in a phosphate ligand are too slight to be significant in this experiment (Reed & Leyh, 1980).

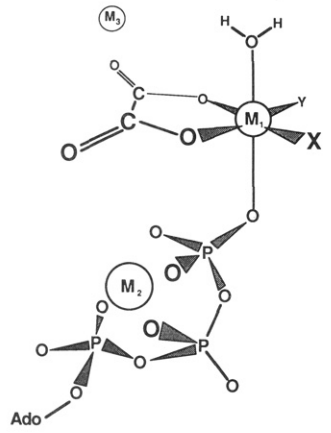

FIGURE 7: Schematic drawing of the active site of pyruvate kinase with oxalate, ATP, and the three inorganic cofactors present. M_1, M_2, and M_3 represent respectively the divalent cation at the protein-based site, the divalent cation at the nucleotide site, and the monovalent cation. The M_2 complex with ATP has been tentatively sketched as a tridentate chelate with stereochemical configurations of the β,γ- and α,β-chelate rings consistent with the data of Dunaway-Mariano and Cleland (1980) and of Jaffe (1979), respectively.

in Figure 6A. The titration experiment shows that signals characteristic of the complex enzyme–Mn(II)–oxalate appear initially, but these signals broaden and diminish in amplitude at higher levels of Mn(II). Amplitudes of the EPR signals diminish even though the total amount of Mn(II) is greater. Spectra that are observed experimentally are superpositions of spectra for all species of Mn(II) present [e.g., enzyme–Mn(II)–oxalate, enzyme–Mn(II)–oxalate–Mn(II)–ATP, and $Mn^{II}ATP$ free in solution]. Although part of the broadening could be attributed to overlap of signals for the different species of Mn(II) present, the fact that the amplitudes of the signals decrease indicates that the component spectra have greater line widths in the enzyme–Mn(II)–oxalate–Mn(II)–ATP species than in the magnetically dilute circumstances. The analogous experiments with Mg(II) and Mn(II) (see Figure 1B) show that occupation of the metal–nucleotide site with a diamagnetic metal ion sharpens signals for the bound Mn(II) at the protein, oxalate site. It is therefore unlikely that broadening of the signals is a structural effect due to binding of $Mn^{II}ATP$ at the nucleotide site. Broadening of the signals is most probably due to spin–spin interaction between the two closely spaced Mn(II) ions. Similar EPR observations have been reported previously for the Mn(II) binding sites on inorganic pyrophosphatase (Banerjee & Cooperman, 1983; Knight et al., 1984).

Spectra for points of a similar titration with ADP replacing ATP are shown in Figure 6B. The results are quantitatively similar to those obtained with ATP present.[5] The dipolar component of the magnetic interaction is sensitive to the distance between the two paramagnetic species (Leigh, 1970). Exchange contributions to the magnetic interaction would also be sensitive to distance (Beuttner & Coffman, 1978). The results, therefore, imply that the Mn(II)–Mn(II) distances do not change appreciably whenever ADP replaces ATP at the active site.

Structural Conclusions. A schematic drawing of the enzyme–Mn(II)–oxalate–Mg(II)–ATP complex is given in Figure 7. Although the EPR data and resulting structure relate to a complex that is a combination of a natural substrate (ATP) and an analogue (oxalate) of the presumed reactive form of pyruvate, there are reasons to anticipate a similar scheme for the corresponding complex with the enolate of pyruvate. Oxalate is a linear, competitive inhibitor of the P-enolpyruvate/pyruvate site on the enzyme (Reed & Morgan, 1974). Binding of oxalate to isozymes (e.g., from liver and from erythrocytes) of pyruvate kinase that exhibit cooperativity in substrate binding triggers the T–R transition that normally occurs upon binding of P-enolpyruvate (Buc et al., 1978). EPR experiments with ^{17}O-enriched pyruvate in the complex with enzyme, Mn(II), and vanadate show that pyruvate is a bidentate ligand for Mn(II) (Lodato, 1986).

The identities of the two ligands from the protein (X and Y) have not been determined for this particular complex. It is likely that X and Y are two of the original three ligands (i.e., the carboxylate of Glu-271 and the carbonyl groups of Ala-292 and Arg-293) implicated as ligands for Mn(II) in the binary enzyme–Mn(II) complex (Muirhead et al., 1986).

The geometrical arrangement of the ligands about Mn(II) in Figure 7 is somewhat arbitrary. Molecular models indicate, however, that a trifacial arrangement of oxygens from oxalate and from the γ-phosphate of ATP involves less strain in a subsequent transition state for phospho transfer than a corresponding meridional arrangement of these three ligands.

The exact mode of coordination between Mg(II) and ATP is not yet established. The Δ epimer of β,γ-bidentate $Cr^{III}ATP$ serves as a substrate for the phosphorylation of glycolate (Dunaway-Mariano & Cleland, 1980). The γ-phosphate group could be coordinated to both divalent ions as in crystals of $Zn^{II}ATP$–2,2′-bipyridyl (Orioli et al., 1980). ^{31}P chemical shifts in the NMR signals for ATP in complexes with enzyme, oxalate, and Mg(II) were consistent with an α,β,γ-tridentate chelate for the $Mg^{II}ATP$ at the active site (Gupta & Mildvan, 1977). The experiments with two Mn(II) ions bound at the active site in the presence of ATP or of ADP indicate that the two paramagnetic ions experience approximately the same magnetic interaction whether ATP or ADP is present. This observation does not necessarily indicate that the $Mn^{II}ATP$ complex is α,β-bidentate. If, however, both ions were coordinated to the γ-phosphate group, one might expect that the bridging γ-phosphate ligand would provide a facile pathway for superexchange interactions between the two manganous ions such as those observed in some complexes with *S*-adenosylmethionine synthetase (Markham, 1981). The absence of such resolved superexchange coupling is not, however, sufficient evidence to rule out a shared γ-phosphate ligand. On the other hand, the similar responses observed with $Mn^{II}ADP$ and with $Mn^{II}ATP$ indicate that the γ-phosphate is not required for the paramagnetic interaction to occur. Clearly, more information is needed to define the coordination scheme for the metal–ATP species.

One carboxylate from oxalate has been sketched as a bridging ligand between Mn(II) and the monovalent cation. This bridging carboxylate scheme is consistent with estimates of the distance between the monovalent and divalent cations obtained from NMR relaxation data (Reuben & Kayne, 1971;

[5] In contrast to the experiment with the diamagnetic complex, $Mg^{II}ADP$, the experiments with ADP and two Mn(II) ions bound sense effects that are due to paramagnetic interactions. ATP is required for the diamagnetic, structural effect, because the γ-phosphate group enters the coordination sphere of Mn(II) at the protein–oxalate subsite. The observations indicate that the two paramagnetic ions are <10 Å apart. In the formulation of the EPR line-shape effects from a dipolar interaction between two spins on a rigid lattice (Leigh, 1970), one of the simplifying assumptions is that one of the spins have a much shorter spin–lattice relaxation time than the other. This assumption is clearly violated in the present case where the two Mn(II) ions have very similar relaxation times. Hence, it is not feasible to use this formalism together with the experimentally observed decrease in the amplitudes of the EPR signals to further quantitate the distance between the two ions.

Hutton et al., 1977; Ash et al., 1978; Raushel & Villafranca, 1980). The scheme retains half of the role for the monovalent cation proposed earlier (Nowak & Mildvan, 1972).

The bifunctional character of the carboxylate group makes it possible that the bidentate coordination of oxalate involves both oxygens from a single carboxylate of the oxalate ligand. Such an "end-on" binding mode for oxalate cannot be rigorously excluded. The magnitude of the zero-field splitting interaction for Mn(II) in this complex is, however, indicative of a nearly cubic electronic environment for the ion. Molecular models show that an end-on binding mode for oxalate would involve substantial geometric distortion that would likely be reflected in much greater anisotropy in the zero-field splitting tensor (Newman & Urban, 1975; Reed & Markham, 1984).

Although the structure for the analogous complex with pyruvate is not yet available, the structure for the complex with oxalate and ATP does offer a "preview" into the stereochemistry of the active site and clues regarding the possible roles for the divalent cations in catalysis. The structure of Figure 7 exhibits an efficient use of the three inorganic cofactors in balancing negative charges of the substrates and in providing points for binding of both substrates. Chelation of a keto acid substrate to M_1 would promote enolization of this species for subsequent nucleophilic attack on the γ-phosphate of ATP. The reactivity of nucleophiles coordinated to metal ions has been demonstrated in model complexes (Buckingham et al., 1969, 1970; Gahan et al., 1985). Simultaneous coordination of M_1 to the attacking nucleophile and to a pendant oxygen from the γ-phosphate of ATP indicates that the coordination sphere of M_1 provides a template for this associative (Blattler & Knowles, 1979) phospho-transfer step. Although the corresponding transition state involves a four-member chelate ring, similar structures have been implicated in reactions of model complexes (Cornelius, 1980; Farrell et al., 1969), and four-membered chelate rings in phosphato complexes have been characterized (Farrell et al., 1969; Haromy et al., 1982).

A scheme in which ATP acts as a bridging ligand between two divalent metal ions is one possible strategy for promotion of phospho transfer between anionic substrates. Other enzymes such as P-enolpyruvate carboxykinase (Foster et al., 1967), pyruvate carboxylase (carboxylation of biotin partial reaction) (Barden & Scrutton, 1974), and glutamine synthetase (Hunt et al., 1975; Balakrishnan & Villafranca, 1978), which are known to require two divalent cations for activation of their catalytic functions, might also exploit a similar scheme with the nucleoside triphosphate as a bridging ligand.

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Acceleration of Surface-Dependent Autocatalytic Activation of Blood Coagulation Factor XII by Divalent Metal Ions†

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ABSTRACT: The effect of divalent metal ions on the rate of dextran sulfate dependent autocatalytic activation of human blood coagulation factor XII was studied at pH 7.4 and 25 °C. Zn^{2+} and Cu^{2+}, but not Co^{2+}, increased the rate of factor XII activation induced by dextran sulfate with optimum effects at approximately 5 and 1 μM, respectively, while Ca^{2+} acceleration required much higher concentrations (millimolar). Further investigation of the effect of Zn^{2+} on factor XII activation demonstrated a complete dependence on the presence of dextran sulfate, lack of inhibition by soybean trypsin inhibitor, the appearance of α-XIIa as the primary reaction product, and reaction kinetics characteristic of an autocatalytic process. These results were consistent with Zn^{2+} affecting only the rate of surface-mediated factor XII autoactivation. The initial turnover velocity of dextran sulfate induced factor XII autoactivation increased linearly with factor XII concentration in the absence of Zn^{2+} up to 0.9 μM factor XII but showed saturation behavior over this same concentration range in the presence of 5 μM Zn^{2+}, indicating that Zn^{2+} increased the reaction rate primarily by lowering the apparent K_m. Comparison of the kinetics of autoactivation at $\mu = 0.15$ and 0.24 revealed that the enhancement in the apparent k_{cat}/K_m brought about by Zn^{2+} increased from 19-fold to 520-fold, respectively, due to a differential dependence of the Zn^{2+}-stimulated and unstimulated reactions on ionic strength. Evidence that enhanced binding of factors XII and α-XIIa to dextran sulfate contributed to the Zn^{2+} rate enhancement was provided by the observation that factors XII and α-XIIa were eluted at higher ionic strengths from a dextran sulfate–agarose column in the presence of 5 μM Zn^{2+} than in its absence. α-Factor XIIa bound more tightly to dextran sulfate–agarose than factor XII in the presence and absence of Zn^{2+}, while β-factor XIIa, which lacks the surface binding domain, did not bind to the column under the same conditions. The rate of dextran sulfate dependent contact activation in normal human plasma, but not in factor XII, prekallikrein, or high molecular weight kininogen deficient plasmas, was stimulated by Zn^{2+}, suggesting a possible role for metal ions in promoting surface-dependent contact activation reactions under physiological conditions as well as in model systems.

Surface-dependent activation of factor XII is thought to be the initial event in the intrinsic blood coagulation cascade (Griffin & Cochrane, 1979). Factor XIIa activates factor XI and prekallikrein in reactions stimulated by surfaces and the protein cofactor HMW-kininogen[1] (Griffin & Cochrane, 1976a). Evidence has been presented that surfaces may participate in factor XII activation by inducing conformational changes in factor XII that enable it to act directly on its protein substrates (Kurachi et al., 1980; Heimark et al., 1980; Ratnoff & Saito, 1979a,b) and by enhancing the susceptibility of factor XII to proteolytic activation (Griffin, 1978; Rosing et al., 1985), as well as by serving as a site for assembly of the proteins involved in these reactions. Surface- and HMW-kininogen-dependent proteolytic activation of factor XII by

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[1] Abbreviations: HMW-kininogen, high molecular weight kininogen; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CK, chloromethyl ketone; PEG, poly(ethylene glycol); DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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