Interaction of Rabbit Muscle Aldolase at High Ionic Strengths with Vanadate and Other Oxoanions†

Debbie C. Crans,* Katakam Sudhakar, and Thomas J. Zamborelli

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523

Received December 26, 1991; Revised Manuscript Received April 13, 1992

ABSTRACT: Reductive, nonreductive, and photolytic interactions of vanadate with fructose-1,6-bisphosphate aldolase were examined and used to explore the interactions of oxoanions with aldolase. Aldolase is known to interact strongly with oxoanions at low ionic strength and weakly at higher ionic strength. Oxoanions inhibit aldolase competitively with respect to fructose 1,6-bisphosphate although the location of the oxoanion binding site on aldolase remains elusive. In this work, the interaction of aldolase with a series of oxoanions was compared at ionic strength approaching physiologic levels. The size and shape of the anion were important for the effective binding to aldolase, and no significant increase in affinity for aldolase was observed by the addition of alkyl groups to the oxoanions. Vanadate competitively inhibits aldolase in a manner analogous to the other oxoanions. Since vanadate solutions contain a mixture of vanadate oxoanions, the nature of the inhibition was determined using a combination of enzyme kinetics and ^{51}V NMR spectroscopy. Aldolase contains a significant number of thiol functionalities, and as expected, vanadate undergoes redox chemistry with them, generating an irreversibly inhibited aldolase. This oxidative chemistry was attributed to the vanadate tetramer, whereas vanadate dimer was a reversible inhibitor. Vanadate monomer does not significantly interact with aldolase reversibly or irreversibly. Vanadyl cation has the lowest inhibition constant under these high ionic strength conditions. Using Yonetani–Theorell analysis, it appears that phosphate, pyrophosphate, and sulfate bind to the same site on aldolase, whereas vanadate, arsenate, and molybdate bind to another site. UV light-induced photocleavage of aldolase by vanadate was examined, and the loss of aldolase activity was correlated with cleavage of the aldolase subunit. Further studies using vanadium as a probe should reveal details on the location of the vanadate and vanadyl cation binding sites. This study suggests several sites on aldolase will accommodate oxoanions, and one of these sites also accommodates vanadyl cation.

The glycolytic enzyme aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase), which catalyzes the reversible aldol cleavage of fructose 1,6-diphosphate (FDP) to triose phosphates [glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP)], is known to interact with oxoanions (Ginsberg & Mehler, 1966; Grazi, 1975; Kasprzak & Kochman, 1980; Koppitz et al., 1986; Matteuzzi et al., 1985; Mehler, 1963; Palczewski et al., 1985; Rose & O'Connell, 1969; Sanchez de Jimenez et al., 1964). Despite significant knowledge about the enzyme reaction mechanism (Rose & Warms, 1985), and the crystal structure at 2.7-Å resolution, the interactions of anions with aldolase are still puzzling (Sygusch et al., 1987). It is known that many organic phosphate compounds such as ATP (Kasprzak & Kochman, 1980; Palczewski et al., 1985), NADP (Matteuzzi et al., 1985), and inositol phosphates (Koppitz et al., 1986) interact strongly and competitively against FDP at low ionic strength. Affinity label studies using radiolabeled 8-azido-ATP showed the ATP binds to Thr-265 (Palczewski & Kochman, 1987) whereas 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine binds to Lys-107 and Tyr-363 (Kasprzak & Kochman, 1980; Palczewski et al., 1985). A sulfate anion also binds in the vicinity of the former site as suggested by the electron density of a sulfate group in aldolase crystallized in a high sulfate containing medium (Sygusch, personal communication). The activity of aldolase is very sensitive to ionic strength, and the K_m for substrate and the K_i's for inhibitors will vary drastically according to specific assay conditions (Rose & O'Connell, 1969). Although tight binding of oxoanions to aldolase at low ionic strength is of mechanistic interest, these interactions may not have physiological relevance unless corresponding interactions also take place at high ionic strength. The present study was carried out to systematically compare the affinity of aldolase for various oxoanions at high ionic strength, compare their binding sites, and thus determine the shapes and nature of anions that interact most strongly with aldolase.

Aqueous solutions of vanadate contain several rapidly exchanging oxoanions including vanadate monomer, dimer, tetramer, and pentamer (Pope, 1983; Pettersson et al., 1985; Crans et al., 1990a,b). Vanadate monomer is a phosphate analog, the dimer is a pyrophosphate analog, and vanadate tetramer and pentamer are cyclic anions (Pettersson et al., 1985). In addition, vanadate decamer is a compact oxoanion that forms at acidic pH (Pope, 1983). Vanadate oxoanions, specifically vanadate dimer, tetramer, and decamer, have been shown to inhibit several glycolytic and pentose phosphate shunt enzymes including dehydrogenases (Crans et al., 1990a,b; Crans & Schelble, 1990; Crans & Simone, 1991), kinases (Boyd et al., 1985), and mutases (Csermely et al., 1985; DeMaster & Mitchell, 1973; Soman et al., 1983). Aldolase interacts strongly with compact and highly charged anions. The specificity for particular vanadate oxoanions, and how such an anion or anions compete with other oxoanions in binding to aldolase, is of mechanistic interest. It is also important to determine whether such anion interactions have physiological relevance.

Aldolase contains several thiol groups (Offermann et al., 1984), some of which are essential for enzyme activity (Kobashi & Horecker, 1967) and may react with vanadate, since van-

† This work was supported by the NIH and Eli Lilly.

0006-2960/92/0431-6812$03.00/0

adate reacts with cysteine to generate cystine and vanadium(IV) (Cohen et al., 1987; Kustin & Toppen, 1973). The presence of thiol groups in the vanadate binding site of an enzyme does not necessarily lead to redox chemistry between vanadate and the enzyme (Crans & Simone, 1991). Nevertheless, reduction of vanadium(V) yields vanadium(IV), which in free form exits as vanadyl cation (VO^{2+}) (Chasteen, 1981). In the past, denatured aldolase has been found to reactivate in the presence of reactivation buffers (Chan et al., 1973). Upon reduction of vanadate, aldolase is presumably oxidized since it can be reactivated using reactivation buffers once the vanadium is removed with EDTA. Vanadium(IV) added in the form of vanadyl cation dichloride was surprisingly found to inhibit aldolase potently, although this form of vanadium presumably exists as a hydrated cation. The interaction between vanadate and aldolase involves redox chemistry and leads to inactivation of aldolase by oxidation of thiol groups; in this work, the irreversible inhibition of aldolase is attributed to the vanadate tetramer.

UV light-induced reaction of vanadate with myosin (Cremo et a., 1990, 1991) and ATPase dynein (Gibbons et al., 1987) has been used for exploration of the vanadate binding site of these enzymes. Ultraviolet irradiation of these enzymes in the presence of vanadate leads to cleavage of the protein, presumably at the binding site, and thus acts as a reported group for binding of the oxoanion. The studies of oxoanions with aldolase would benefit from experiments that identified the interaction sites of the anion. The UV light-induced cleavage experiments were explored successfully with aldolase despite the irreversible interaction between vanadate and aldolase. The specific cleavage sites await identification by sequencing. The application of vanadate oxoanions described in this work has assisted and further promises to assist in the characterization and understanding of the affinity of aldolase for oxoanions.

MATERIALS AND METHODS

Reagents, Stock Solutions, and Enzymes. The reagents used were reagent grade. Water was distilled and then deionized on an anion-exchange column. Vanadium pentoxide and vanadyl(IV) oxide were purchased from Fisher Scientific and Aldrich. Sodium molybdate and sodium arsenate were obtained from J. T. Baker, and tungstic acid was from Eastman Kodak. The enzymes and biochemicals were purchased from Sigma. A vanadate stock solution was prepared by dissolving vanadium pentoxide with 2 equiv of sodium hydroxide to generate a vanadate solution of 0.25 M; this solution was stored at 4 °C. The concentrations of the vanadate standard solution were monitored by UV spectroscopy (ϵ_{265} = 3550 M^{-1} cm^{-1}) (Newman et al., 1958). No changes in concentrations were observed over the course of 6 months. The vanadyl cation stock solution was prepared by dissolving appropriately weighed vanadium(IV) oxide into 0.1 M HCl. The stock solutions were kept at 4 °C. Since the pH was <2, we observed no oxidation of the vanadyl cation. Aldolase (EC 4.2.1.13) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) were from rabbit muscle. The enzymes were used directly or dialyzed after most of the ammonium sulfate had been removed by precipitation of the protein in an ammonium sulfate suspension and decanting of the supernatant. No differences were observed in the kinetic data with enzymes that had been dialyzed or used directly after removal of the ammonium sulfate.

Kinetic Measurements. Spectrophotometric determinations of rates of cleavage of FDP were measured at 25 °C and 340 nm on a Lambda 4B Perkin-Elmer double-beam spectrophotometer equipped with a constant-temperature cell. Controls were run before and after each series of constant inhibitor concentration, and the rates were corrected if necessary. The protein concentrations were quantified by the method of Lowry (Lowry et al., 1951).

^{51}V NMR Spectroscopy. ^{51}V NMR spectra were recorded at 52 MHz on a ^{1}H 200-MHz Bruker WPSY (4.7 T) spectrometer. We typically used spectrum widths of 8064 Hz, a 90° pulse angle, and no relaxation delay. No changes were observed in integration of various peaks if the relaxation delay was increased. The chemical shifts are reported relative to the standard $VOCl_3$ (0 ppm) although in practice a solution of vanadate and diethanolamine at pH 8.8 was used (Crans & Shin, 1988). The spectra were recorded using external lock. The NMR samples were prepared as described for the assay with the exception that NADH or FDP were omitted. It was shown experimentally that omission of cofactors and substrate would not noticeably affect the distribution of vanadate species since results of samples including these species were within a 5% experimental uncertainty.

Speciation Analysis. Vanadate solutions contain complex mixtures of mono- and oligovanadates that vary with ionic strength, concentration, pH, temperature, and buffer (Pope, 1983; Crans & Shin, 1988). The presence of vanadate oligomers in the assay solutions necessitates the determination of the vanadium species in solution, that is, to perform a speciation analysis. The concentrations of vanadate species were determined in this specific assay solution at pH 7.1 with a known amount of total vanadium. Vanadate monomer (–555 ppm), dimer (–569 ppm), tetramer (–574 ppm), and pentamer (–582 ppm) are related as shown by eq 1–4 as described

$$2V_1 \overset{K_{12}}{\rightleftarrows} V_2 \quad (1)$$

$$4V_1 \overset{K_{14}}{\rightleftarrows} V_4 \quad (2)$$

$$5V_1 \overset{K_{15}}{\rightleftarrows} V_5 \quad (3)$$

$$2V_2 \overset{K_{24}}{\rightleftarrows} V_4 \quad (4)$$

previously. The H^+-dependent equilibrium constants for this assay were determined to be $K_{12} = 4.6 \times 10^2$ M^{-1}, $K_{14} = 6.0 \times 10^9$ M^{-3}, $K_{24} = 2.8 \times 10^4$ M^{-1}, and $K_{15} = 9.9 \times 10^{11}$ M^{-11}, in agreement with values published previously under similar conditions (Crans & Schelble, 1990).

Aldolase Assay. Given the chemical reactions of vanadate with common buffers and compounds present in the aldolase assay (specifically DHAP), we only report studies in the direction of cleavage, as well as use of modified assay of the combined triosephosphate isomerase (TIM) and glycerol-3-phosphate dehydrogenase (G3PDH) assay.

(A) Vanadate Inhibition Studies. The assay solutions contained 50 mM imidazole, 400 mM KCl, 0.20 mM NADH, and 0.027 mg of aldolase at pH 7.10 and 25 °C. FDP concentration was varied from 0.010 to 0.25 mM. The assay solutions were incubated for 3 min, then vanadate was added, and the reaction was started by the addition of enzyme. The initial rates were monitored for 45 s at 340 nm. Rates were measured in the presence of 0.0–3.5 mM vanadate.

(B) Anion Inhibition Studies. Analogous conditions were used when examining the inhibition by phosphate, pyrophos-

phate, molybdate, tungstate, arsenate, sulfate, Zn^{2+}, and vanadate decamer. Ionic strength was maintained by adding 400 mM KCl at 0 mM inhibitor concentration. Increases in inhibitor concentrations were compensated for by proportionally decreasing the KCl concentration. The phosphate concentration varied from 0.0 to 50 mM, pyrophosphate from 0.0 to 20 mM, molybdate from 0.0 to 25 mM, arsenate from 0.0 to 7.0 mM, Zn^{2+} from 0.0 to 2.0 mM, sulfate from 0.0 to 80 mM, and decamer from 0.0 to 2.5 mM.

(C) Vanadyl Cation Inhibition Studies. The conditions were as described above for vanadate inhibition studies, except all solutions were purged a minimum of 2 h with nitrogen before use. The assay was carried out under nitrogen in a cuvette with a rubber stopper to limit oxygen diffusion into the cuvette.

UV Light-Induced Vanadate Cleavage Experiments. Aldolase (1 mg/mL) in 10 mM Tris, 5.0 mM $MgCl_2$, and 100 mM KCl at pH 7.5 was incubated at 4 °C for 30 min in the presence of 2.0 mM vanadate. The enzyme solution was irradiated at 365 nm for various times (0, 1, 2.5, 5, 7.5, 10, 15, 20, 30 min) with UV light using a Mineralight UV lamp Model UVGL-25 (in longwave mode), placed 3 cm from the aldolase sample. The aldolase was removed after irradiation and assayed at 340 nm in the presence of excess EDTA using the assay described above.

Aldolase irradiated with and without vanadate was denatured in 4% SDS and 10% β-mercaptoethanol at 90 °C for 30 min. The denatured samples were loaded on a 12% SDS–polyacrylamide gel (Laemmli, 1970) and run for 4 h at constant current (25 mA). The gel was removed and stained overnight in 0.125% Coomassie Blue R, 50% methanol, and 10% acetic acid. After being destained for 8 h in 50% methanol and 10% acetic acid, the gel was dried for analysis by densitometry.

Densitometry. Densitometry was performed using a Microscan 1000 2-D gel analysis system with a Hewlett-Packard ScanJet Plus accessory. We thank Professor James Bamburg for allowing the use of this system.

Data Analysis. Rates were measured using the initial linear portion of the rate profile. The kinetic data were analyzed by using Cricket graph and Statmac programs for statistical manipulations on the Apple computer. Both Lineweaver–Burk and Eisenthal–Cornish–Bowden types of plots were used to determine Michaelis–Menten parameters. The K_i's and the nature of inhibition were determined from plots of Lineweaver–Burk slopes as a function of the concentration of inhibitor. The fits of the experimental points were determined with cricket Graph and Statmac. The uncertainties indicate the standard deviation of the data fit.

RESULTS AND DISCUSSION

Modified Aldolase Assay. Aldolase was assayed in the cleavage direction using a modified enzyme assay designed to minimize the chemical reactions between vanadate and aldolase. First, imidazole was chosen as buffer, because it is the only buffer that is reported not to facilitate reduction of vanadate (Erdmann et al., 1979; Vyskocil et al., 1980; Ramasarma et al., 1981; Crans & Shin, 1988). Although buffers such as Tris and Hepes are used in many studies, vanadate in these buffers also shows a modest level of reduction under normal assay conditions. We chose to use imidazole in these studies.

Aldolase is assayed by observing the cleavage of fructose 1,6-diphosphate (FDP) to form glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), which are then reduced by glycerol-3-phosphate dehydrogenase (G3PDH) while NADH is oxidized (Horecher et al., 1973). Commonly, the G3P is converted to DHAP by triosephosphate isomerase (TIM), which is then also reduced by G3PDH. The reverse reaction is assayed by radiolabeling either DHAP or G3P and observing the appearance of the label in FDP. Studies of aldolase in the condensation reaction would require the use of variable concentrations of both G3P and DHAP. The compounds in aldolase assays that have the greatest affinity for vanadate are DHAP and dihydroxyacetone (DHA) (Drueckhammer et al., 1989). Both DHAP and DHA will immediately reduce vanadate at neutral pH upon mixing. Kinetic studies of aldolase in the condensation direction would be performed while the redox chemistry between vanadate and DHAP occurs, depending on the concentrations of vanadate and DHAP, respectively. We therefore chose to study aldolase in the cleavage direction, where the concentration and lifetime of DHAP in solution would be minimized.

To further decrease the problem of vanadate exposure to DHAP, we explored the possibility of using an assay based on aldolase coupled with excess G3PDH. Such an assay would measure the concentration of FDP that cleaves to form G3P and DHAP. DHAP would immediately be reduced to glycerol 3-phosphate while oxidizing NADH, whereas G3P would remain in the solution. It has been previously shown that several aldehydes at millimolar concentration slightly stimulate aldolase activity (Spolter et al., 1965). Such levels would not be reached in our assays. One molecule of FDP would result in the oxidation of one molecule of NADH in the modified assay, contrary to the usual assay in which one molecule of FDP results in two molecules of oxidized NADH. This modified assay would decrease the concentration of DHAP in the assay, as well as the time the DHAP has to react with vanadate.

We compared the results of the modified assay with that of the TIM–G3PDH coupling assay using imidazole as buffer and an ionic strength of 0.4. The K_m for the modified assay for FDP was found to be 0.087 mM and the V_{max} 1.00 $mmol^{-1}$ min^{-1}, whereas the TIM–G3PDH assay gave a K_m of 0.090 mM and a V_{max} 1.00 $mmol^{-1}$ min^{-1}. Commercially available aldolase contains less than 0.1% TIM, so that reaction rates by such an impurity will be small. The rates in the TIM–G3PDH assay were approximately twice as high as those in the modified assay, as expected, since the former generated two molecules when the latter generated one. The K_m for F1P for the modified assay was found to be 19 mM and the V_{max} 0.080 $mmol^{-1}$ min^{-1}, whereas the TIM–G3PDH assay gave a K_m of 18 mM and a V_{max} 0.088 $mmol^{-1}$ min^{-1}. In this case, the rates by both assays are very similar since the unphosphorylated portion of the substrate is glyceraldehyde. These results compare favorably with the literature (Rose & O'Connell, 1969). The two assays clearly give compatible results, and the complicating vanadate reactions warrant our use of the modified assay in studies involving vanadate.

Vanadate undergoes a series of reactions under assay conditions, and these reactions are very sensitive to ionic strength, pH, temperature, and assay components (Pope, 1983; Gresser & Tracy, 1985; Crans & Shin, 1988). We will therefore conduct our studies at high ionic strength, such that the distribution of vanadate derivatives will be kept constant, and our observations will indeed be due to the interaction of aldolase with specific vanadate derivatives. We chose to carry out all of our kinetic studies at 0.4 ionic strength because this approximates the ionic strength at physiological conditions.

Inhibition of Aldolase by Oxoanions. The inhibition studies with phosphate, pyrophosphate, arsenate, molybdate, and tung-

Table I: Inhibition Constants in FDP-Catalyzed Cleavage by Aldolase A from Rabbit Muscle[a]

inhibitor	inhibition pattern	K_i (mM)	uncertainties[c]
phosphate	competitive	19.5[b]	±1.3
pyrophosphate	competitive	12.8[b]	±1.0
sulfate	competitive	35[b]	±6.7
arsenate	competitive	6.10[b]	±0.29
molybdate	competitive	4.28[b]	±0.49
tungstate	competitive	2.10[b]	±0.31
fructose 6-phosphate	competitive	23.1[b]	±0.8
V_{i2}	competitive	0.23[b] or 0.46[b] V atoms	±0.025, ±0.10
V_{i4}	competitive (irreversible)	0.52[b] or 2.08[b] V atoms	±0.02, ±0.08
		1.08[d] or 4.32[d] V atoms	±0.05, ±0.20
V decamer	noncompetitive	0.92 or 9.20 V atoms	±0.29
vanadyl cation	competitive	0.083[b]	±0.006
Zn^{2+}	noncompetitive	1.49[b]	±0.04
$[(CH_3)_2AsMo_4O_{14}OH]^{2-}$	competitive	0.56[b]	±0.06
$[(R_2P)_2Mo_5O_{21}]^{4-}$	competitive	0.73[b]	±0.03

[a] The assay is carried out in 0.10 M imidazole and 0.40 M KCl at pH 7.3 and 25 °C in the presence of G3PDH. [b] K_m = 0.087 mM, and K_i is defined as $[E][I]/[E{\cdot}I] = k_{-1}/k_1$. [c] The uncertainties represent calculated standard deviations. We used the program Statworks to calculate the standard errors. [d] K_i is defined as $[E][V_4]/[E{\cdot}V_4] = (k_{-1} = k_2)/k_1$, where k_2 = 0.47 ● 0.02 min^{-1} at 15 °C. In this case, K_{i4} is a kinetic constant as described by Briggs and Haldane (Segel, 1975).

state were carried out in 0.10 M imidazole, 0.40 M KCl, 0.20 mM FDP, excess G3PDH (0.021 mg, about 4.5 unit), and aldolase (about 0.02 mg) at pH 7.1. These oxoanions were found to be competitive inhibitors (Table I). In general, they are much weaker inhibitors than reported previously at low ionic strength. We attribute the high inhibition constants to the high ionic strength, since our observations parallel inhibition studies reported at higher ionic strength (Mehler, 1963; Sanchez de Jimenez et al., 1964; Rose & O'Connell, 1969). Phosphate is a better inhibitor than sulfate but worse than pyrophosphate. Even in this high ionic strength medium, a correlation between anionic charge and strength of inhibition was observed analogous to those reported at low ionic strength. Arsenate is a better inhibitor than phosphate, but worse than either molybdate or tungstate, suggesting that the charge of the inhibitor is not the only factor of importance when binding to aldolase.

In addition to examining simple oxoanions, the effects of two cyclic heteropolyanions were explored. These two cyclic molybdate derivatives were chosen because they are structurally similar to vanadate tetramer (Barkigial et al., 1975) and pentamer (Stalick & Quicksall, 1976). The cyclic molybdate tetramer, $(CN_3H_6)_2[(CH_3)_2AsMo_4O_{14}(OH)]{\cdot}H_2O$, and pentamer, $[(CH_3)_3N]_4[(C_6H_5P)_2Mo_5O_{21}]{\cdot}5H_2O$, were both competitive inhibitors. The K_i for the tetramer was 0.56 (±0.6) mM, and the K_i for the pentamer was 0.73 (±0.03) mM. Both large oxoanions were more potent inhibitors than the simpler oxoanions described above.

Despite the observation that all of the oxoanion inhibitors are competitive, and that such a pattern would be consistent with the binding of the anion of the substrate binding site, such interpretation may be erroneous. Crystallographic studies of aldolase with sulfate at high ionic strength have shown that sulfate interacts with aldolase at a site on the interface between two aldolase subunits (Sygusch, personal communication). This site also binds ATP at low ionic strength as shown by labeling studies with 8-azido-ATP (Palczewski et al., 1985). Fructose 6-phosphate was found to be a worse inhibitor than phosphate against FDP. This result suggests that no additional stabilization of the aldolase inhibitor complex was obtained by adding the fructose to the phosphate. Such an observation would support the interpretation that the oxoanions bind to the subunit–interface site, because no additional binding affinity is obtained when the inhibitor looks more like the substrate. We will use vanadate to obtain information on the preferred structure of the vanadate oxoanions that interact with aldolase, as well as using this oxoanion as a photocleavable agent to define the interaction between oxoanions and aldolase.

The high inhibition constants for the simple but physiologically significant oxoanions such as phosphate, pyrophosphate, and organic phosphates suggest that aldolase is not likely to be significantly inhibited by these anions under physiological conditions. Although the anions with higher charges (such as pyrophosphate compared to phosphate or ATP compared to ADP) are stronger inhibitors than the less charged species, the inhibition constants are still significantly higher than the physiological concentrations of these compounds. One would therefore not expect these metabolites to significantly inhibit aldolase under normal conditions. High ionic strength, however, will reduce the overall aldolase activity by increasing the K_m for the substrates and decreasing the V_{max}. The latter effect is a composite effect from all anions in the cellular environment and presumably involves the interactions of various anions with different sections of aldolase; characterization of various anion interactions with aldolaase will therefore contribute to understanding the physiological modes of action of this enzyme.

Inhibition of Aldolase by Vanadate. Aldolase is inhibited by vanadate in solutions containing 0.10 M imidazole, 0.40 M KCl, 0.20 mM FDP, excess G3PDH (0.02 mg), and aldolase (about 0.02 mg) at pH 7.1. Since aldolase contains several sulfhydryl groups, it is possible the vanadate interacts with aldolase through redox chemistry. The reversibility of the vanadate inhibition was therefore determined first.

To an assay solution containing FDP, NADH, and G3PDH were added various amounts of vanadate, and the assay was started with aldolase; the rate decreased according to the vanadate concentrations. When 1 equiv of EDTA was added, an EDTA–vanadate complex forms. However, no significant rate increases were observed in the assay if EDTA was added 3–10 min after the reaction was started. If aldolase was incubated with vanadate 2 min or less prior to starting the assay with FDP, the addition of EDTA did show noticeable rate increases, through the rates never returned to the control levels when no vanadate was added. Since the removal of vanadate did not generate rates analogous to rates in assays without vanadate, vanadate interacts irreversibly with aldolase. Various concentrations of EDTA were added to assays in which aldolase and vanadate had been incubated for 2 min and various degrees of reactivation were observed. Reactivation of aldolase by removing vanadate by EDTA suggests that vanadate interacts reversibly with aldolase. We conclude vanadate interacts both reversibly and irreversibly with aldolase.

Solutions of vanadate contain several oxoanions, of which several have previously been reported to interact with enzymes. Vanadate tetramer inhibits strongly 6-phosphogluconate dehydrogenase (Crans et al., 1990a,b). Glucose-6-phosphate dehydrogenase (Crans & Schelble, 1990) and glycerol-3-phosphate dehydrogenase (Crans & Simone, 1991) are inhibited by both the dimer and the tetramer. Vanadate decamer inhibits phosphofructose kinase and hexokinase (Boyd et al., 1985). Since vanadate interacts both reversibly and

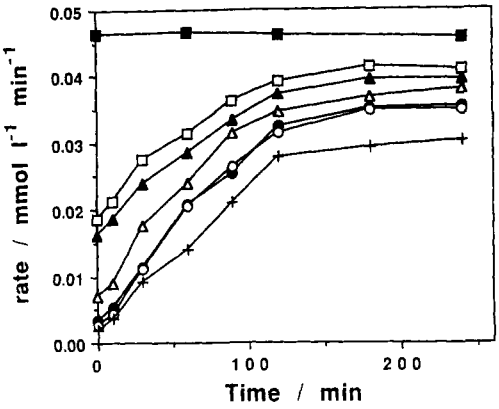

FIGURE 1: Reactivation of aldolase denatured by denaturation buffer and vanadate as a function of time. The rates of FDP cleavage and corresponding NADH oxidations were determined of the following samples: (1) control (aldolase in 0.10 M imidazole buffer at pH 7.1) (■); (2) aldolase denatured using 6 M guanidine hydrochloride, 0.10 M imidazole, pH 7.1, 1.0 mM EDTA, and 10 mM β-mercaptoethanol and incubated for 2 h; the renaturation buffer contained 50 mM imidazole pH 7.1, 1.0 mM EDTA, 0.14 M β-mercaptoethanol, 1.0 mg/mL BSA, and 20% sucrose (●); (3) aldolase denatured using the above denaturation buffer for 2 h was added to the renaturation buffer containing 4 mM EDTA (○); (4) aldolase denatured with 3.0 mM vanadate for 2 h and reactivated using the renaturation buffer containing 1 mM EDTA (+); (5) aldolase denatured with 3.0 mM vanadate for 2 h and reactivated using the renaturation buffer containing 4 mM EDTA (△); (6) aldolase denatured with 2.0 mM vanadate for 2 h and reactivated using the renaturation buffer containing 4 mM EDTA (▲); (7) aldolase denatured with 3.0 mM vanadate for 1 min and reactivated using the renaturation buffer containing 4 mM EDTA (□).

irreversibly with aldolase, it is possible that one oxoanion interacts both reversibly and irreversibly with aldolase or that one oxoanion is responsible for the reversible and another for the irreversible vanadate activity. In order to examine these possibilities, we quantified the irreversible interaction of vanadate with aldolase.

Irreversible Inhibition of Aldolase by Vanadate. The vanadate-induced irreversible inhibition of aldolase could be a result of vanadate reacting with thiol groups or with the peptide backbone of the enzyme. To examine these possibilities, aldolase was incubated for 2 h with 3 mM vanadate such that the enzyme was completely inhibited by vanadate. Both aldolase with and without vanadate were denatured in 4% SDS and 10% β-mercaptoethanol at 90 °C for 30 min. A 12% SDS–polyacrylamide gel was run, and the gel traces of the vanadate-treated aldolase were compared to the gel traces of untreated aldolase. No sign of peptide cleavage was observed in the gels, suggesting that vanadate does not affect peptide cleavage. It is therefore likely that vanadate reacts with the thiol groups in aldolase.

Aldolase has previously been shown to regain 85% activity in the course of 2 h in a reactivation buffer (50 mM Tris-HCl, 0.14 mM β-mercaptoethanol, 1 mM EDTA, 1 mg/mL BSA, and 20% sucrose at pH 7.5) (Chan et al., 1973). If the irreversible inhibition of aldolase by vanadate is a result of thiol group oxidation, it should be possible to reactivate aldolase using the above reactivation buffer. Inactivated aldolase was reactivated by the guanidine hydrochloride, EDTA, and β-mercaptoethanol mixture, respectively (Figure 1). Aldolase deactivated by vanadate will, after removal of the vanadate, also regain 85% activity in the course of 2-h incubation in reactivation buffer. Both vanadate and vanadyl cation form a vanadium–EDTA complex containing one vanadium and one EDTA. Since vanadate must be complexed completely in order to reactivate all of the aldolase, excess EDTA must

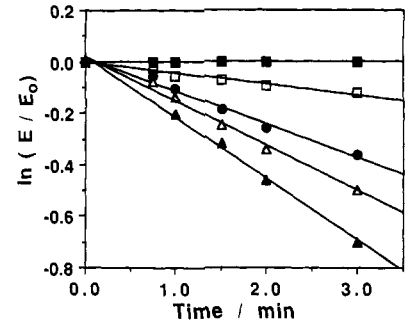

FIGURE 2: Rate of FDP cleavage at 15 °C is plotted as a function of time at various total vanadate concentrations: (■) 0.0, (□) 0.9, (●) 1.5, (△) 2.0, and (▲) 3.0 mM.

be added to the reactivation buffer. Mercaptoethanol in the reactivation buffer will reduce vanadate upon contact and generate vanadyl cation, a potent inhibitor of aldolase (see below); vanadyl cation at millimolar concentrations will complex various components of the assay and not result in millimolar concentrations of free vanadyl cation. Aldolase completely inactivated by vanadate was reactivated by the modified reactivation buffer containing excess EDTA, whereas the reactivation buffer containing 1 mM EDTA did not remove all the inhibitory vanadium (Figure 1). A reactivation buffer containing 1 mM EDTA reactivated aldolase about 10% less than a reactivation buffer containing 4 mM EDTA in 2 h. Since 3 mM vanadate was used to irreversibly inhibit the enzyme, 1 mM EDTA only reduced the vanadate concentration to 2 mM, which could continue to inhibit aldolase in the form of either vanadate or free vanadyl cation. Aldolase incubated with 2.0 mM vanadate for 2 h will have lost about 65% activity. Since this aldolase had a higher initial activity when placed in the reactivation buffer, it regains activity up to the 85% activity level after 90 min. Aldolase incubated with 3.0 mM vanadate for only 1 min contains about 40% activity; such aldolase will, when placed in the modified reactivation buffer, regain the original 85% activity level after 70 min and faster than aldolase which was completely inactivated (Figure 1).

Rates of Irreversible Interaction of Vanadate with Aldolase. The rate of vanadate-induced aldolase inactivation must be determined in order to examine the reversible interaction of vanadate and aldolase. The irreversible vanadate-induced inactivation is measured as the inhibition which could not be reversed by addition of excess EDTA in the assay solution. Vanadate incubated at 25 °C with aldolase reacts very rapidly; the reaction is almost complete within a 2-min period, and no significant reduction in the enzyme activity is observed upon longer (to 30 min) incubation times. Since the rates are too fast to be measured accurately at 25 °C, the reaction of vanadate with aldolase was measured at 15 °C, where the reaction rate was half. In the presence of a large excess of vanadate, vanadate reacts with aldolase according to pseudo-first-order kinetics during the observable parts of the reaction. At 2 mM vanadate concentrations, the enzyme did not completely lose activity in 20 min although the changes at these low rates are within experimental uncertainties. The plots of the logarithm of residual activity of aldolase versus time of incubation with vanadate are linear as predicted by eq 5 (Figure 2) (Palczewski et al., 1985; Kitz & Wilson, 1962).

$$\ln (E/E_0) = -k_{obs}t \quad (5)$$

E and E_0 represent the activity at time t and zero time, respectively. The observed first-order rate constant for the loss of enzymatic activity, k_{obs}, is directly proportional to the concentration of aldolase

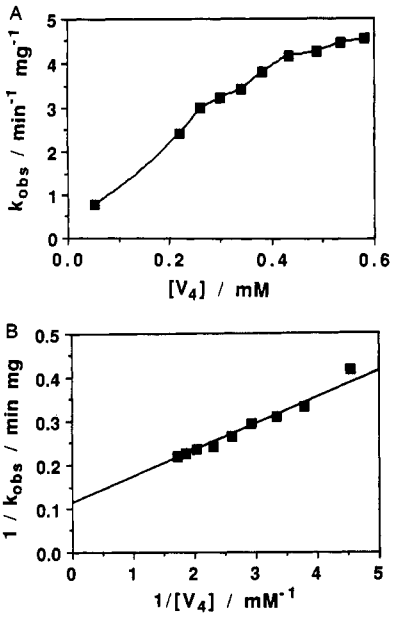

FIGURE 3: Dependence of k_{obs} on [V_4]. (A) Plot of k_{obs} as a function of [V_4]; (B) double-reciprocal plots of $1/k_{obs}$ as a function of $1/[V_4]$.

(data not shown). Little inactivation occurs when vanadate concentrations below 1.5 mM were added to aldolase (Figures 2 and 3A,B). Above 1.5 mM vanadate, the inactivation rates and k_{obs} increase rapidly. Since vanadate solutions contain a mixture of vanadate oligomers, additional information on the interaction of specific vanadate oxoanions with aldolase is required before the rate constant of aldolase deactivation should be further specified.

No irreversible inhibition was observed at total vanadate concentrations below 0.5 mM, suggesting that vanadate monomer was not the irreversible vanadate inhibitor (Crans et al., 1990a,b). At total vanadate concentrations from 0.5 to 0.8 mM, vanadate dimer is present at higher concentrations than vanadate tetramer. Vanadate at 0.6, 0.7, and 0.9 mM concentrations was incubated with aldolase at 15 °C as a function of time, and the residual activity was determined in an assay containing excess EDTA. No significant irreversible inhibition was observed in the incubations of up to 0.9 mM total vanadate (Figure 2), suggesting the vanadate dimer may not be the vanadate derivative responsible for the irreversible modification of aldolase. Above 0.9 mM, the vanadate tetramer is present at higher concentrations than vanadate dimer, and at 2.5 mM, the vanadate pentamer appears in the solutions. Measuring k_{obs} for vanadate concentrations from 0.9 to 3.0 mM shows significant increases in k_{obs} in this vanadate concentration range. At 2.0 mM vanadate, aldolase had from 30 to 40% activity, whereas at 3.0 mM vanadate, aldolase is completely inactivated. From 2.0 to 3.0 mM total vanadate, the tetramer concentration increases significantly, suggesting that the tetramer may be responsible for the irreversible inhibition of aldolase.

This interpretation is examined by plotting k_{obs} (or reciprocal k_{obs}) as a function of tetramer concentration (or reciprocal tetramer concentration) as illustrated by plots in Figure 3A,B. Since no linear relationship is observed between k_{obs} and either dimer, tetramer, or pentamer (see Figure 3A), this suggests aldolase is not simply irreversibly inhibited by any of these anions. As shown in Figure 3B, the increases in the reciprocal k_{obs} correlate linearly with the increases in reciprocal tetramer concentration, suggesting an intermediate protein–tetramer complex forms before inactivation (Palczewski et al., 1985). Corresponding plots with vanadate dimer or pentamer did not give equally reasonable fits. As shown by eq 6, k_2

$$E + V_4 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E{\cdot}V_4 \xrightarrow{k_2} E^* \qquad (6)$$

describes the first-order rate constant of tetramer enzyme complex inactivation. The notation E* describes the irreversibly modified and presumed inactive enzyme. From the intercept in Figure 3B and a molecular mass of 161 kDa for aldolase, k_2 was calculated to be 0.47 min^{-1} at 15 °C. Since [V_4] $\gg E_0$, the relationship between the k_{obs} and the active inhibitor, V_4, is shown in eq 7 (Kitz & Wilson, 1962). In eq 7, [V_4] describes the concentration of vanadate tetramer, and K_{i4} is the inhibition constant defined by eq 6 and 8. Using

$$\frac{1}{k_{obs}} = \frac{1}{k_2} + \frac{K_{i41}}{k_2[V_4]} \qquad (7)$$

$$K_{i41} = \frac{[V_4][E]}{[E{\cdot}V_4]} = \frac{k_{-1}}{k_1} \qquad (8)$$

the data shown in Figures 2 and 3, a K_{iV41} of 0.53 (±0.02) mM vanadate tetramer corresponding to 2.06 (±0.08) mM vanadium atoms was obtained. We conclude that vanadate tetramer is the major vanadate oxoanion responsible for the irreversible inhibition and that a reversible aldolase–tetramer complex is formed before the inactivation occurs. It is of interest to note that this K_i is very similar to that observed for the cyclic molybdate tetramer.

Rates of irreversible modification were determined at 15 °C because the inhibition reaction proceeded too quickly to measure accurately at 25 °C. The rate constant for irreversible inactivation at 15 °C must therefore be extrapolated to 25 °C to be used for determination of the reversible inhibition by vanadate at 25 °C. Assuming the inactivation reaction is a second-order reaction proportional to the tetramer concentration and the aldolase concentration, and that the Arrhenius parameter for this reaction is linear from 15 to 25 °C, we calculate the $k_{2,25}$ as 0.9 min^{-1}.

Inhibition of Aldolase by Vanadyl Cation. In a reducing environment, vanadate produces vanadium(IV). Since we are exploring the effect of vanadium-derived compounds on aldolase, the effects of vanadyl cation must be determined. Vanadyl cation undergoes complex chemistry at neutral pH. Therefore, vanadyl cation is kept in an acidic stock solution and added to completely deoxygenated assay solutions seconds before the assay is to be started. In an assay carried out with these experimental precautions, vanadyl cation was found to be a potent inhibitor at high ionic strength (K_i of 0.083 mM).

Vanadyl cation showed a competitive inhibition pattern analogous to the other vanadate oxoanions studied. The inhibition by vanadyl cation was reversible, because addition of various concentrations of EDTA reversed inhibition by the cation; addition of EDTA in a 1:1 ratio of vanadyl cation completely reversed the inhibition. No inhibition was observed if a solution of vanadyl cation premixed with EDTA at a ratio 1:1 was added to aldolase. In the past, vanadyl cation has been used as an analog for Zn^{2+}. Zn^{2+} was reported as an inhibitor for aldolase (Kobashi & Horecker, 1967), and we find it is a weak noncompetitive inhibitor with a K_i 500-fold larger than that of vanadyl cation. Thus, the interaction of Zn^{2+} and VO^{2+} with aldolase seems significantly different. Vanadyl cation has a high affinity for sulfhydryl ligands, and it is possible that a sulfhydryl group interacts with vanadyl cation, resulting in a tight vanadyl cation–aldolase complex.

Reversible Inhibition by Vanadate Oxoanions. Initial rate measurements at various FDP concentrations and various vanadate concentrations in 0.10 mM imidazole and 0.40 M KCl at pH 7.1 were made within 30 s of mixing the vanadate with aldolase. The rates followed the characteristic competitive inhibition pattern in a Lineweaver–Burk plot. Since no EDTA was added to these assays, inhibition was the result of both irreversible and reversible inhibition by vanadate. As demonstrated above, vanadate tetramer is both a reversible and an irreversible inhibitor; whether other vanadate anions affect aldolase must be determined. No significant inhibition is observed below 0.5 mM total vanadate, suggesting vanadate monomer is not a significant reversible inhibitor. If one vanadate oxoanion is the major inhibiting species, the observed inhibition should correlate linearly with such an anion. No such relationship was observed. Two alternative mechanisms of inhibition are described below, using dimer and tetramer as examples of inhibiting oxoanions; any combination of two inhibiting vanadate oxoanions is possible and has been tested. If vanadate tetramer is the only inhibiting anion, and it is both a reversible and an irreversible inhibitor, the reaction scheme for the rate measurements could be described by eq 9. The K_i is defined as shown by eq 10 and the reciprocal rate by eq 11 in accord with the Michaelis–Menten equation and steady-state considerations.

$$\begin{gathered} E + FDP \underset{k_{-s1}}{\overset{k_{s1}}{\rightleftarrows}} E{\cdot}FDP \xrightarrow{k_p} P \\ E + V_4 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E{\cdot}V_4 \xrightarrow{k_2} E^* \end{gathered} \tag{9}$$

$$K_{iV4} = \frac{[E][V_4]}{[E{\cdot}V_4]} = \frac{k_{-1} + k_2}{k_1} \tag{10}$$

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[V_4]}{K_{iV4}}\right)\frac{1}{[FDP]} \tag{11}$$

It is possible that vanadate tetramer is both a reversible and an irreversible inhibitor, while the dimer is a reversible inhibitor. The inhibition scheme will be described by eq 12, and two K_i's by eq 10 and 13, and the reciprocal rate by eq 14. Substituting $[V_4]$ for $K_{24}[V_2]^2$ yields eq 15.

$$\begin{gathered} E + FDP \underset{k_{-s1}}{\overset{k_{s1}}{\rightleftarrows}} E{\cdot}FDP \xrightarrow{k_p} P \\ E + V_4 \underset{k_{-14}}{\overset{k_{14}}{\rightleftarrows}} E{\cdot}V_4 \xrightarrow{k_2} E^* \\ E + V_2 \underset{k_{-12}}{\overset{k_{12}}{\rightleftarrows}} E{\cdot}V_2 \end{gathered} \tag{12}$$

$$K_{i2} = \frac{[V_2][E]}{[E{\cdot}V_2]} \tag{13}$$

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[V_2]}{K_{iV2}} + \frac{[V_4]}{K_{iV4}}\right) \tag{14}$$

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[V_2]}{K_{iV2}} + \frac{K_{24}[V_2]^2}{K_{iV4}}\right) \tag{15}$$

In order to distinguish between the two inhibition schemes shown in eq 9 and 12, we examined the Lineweaver–Burk slopes and their dependence on the vanadate oligomer concentration. Figure 4 shows the plot of the slopes as a function of vanadate tetramer concentration, suggesting that the fit with the tetramer is almost linear with the exception of the inhibition at low tetramer concentrations. Therefore, tetramer alone cannot explain the observed inhibition dependence of vanadate concentrations. The stronger inhibition at low tetramer concentrations implies that an oligomer present in this vanadate concentration range, such as the dimer, is an active inhibitor. Fitting the data to eq 14 and 15 gives a K_{iV2} of 0.41 ± 0.36 mM dimer (0.82 mM vanadium atoms) and a K_{iV41} of 0.75 ± 0.30 mM tetramer (3.00 mM vanadium atoms). The K_{iV41} calculated in this treatment (defined by eq 10) is comparable to the K_{iV4} of 0.52 ± 0.02 mM calculated using the inactivation studies described by eq 6–8. Since both K_{iV4}'s are within experimental error, these results suggest K_{iV41} approximates k_{iV4} and that $k_{-1} \gg k_2$.

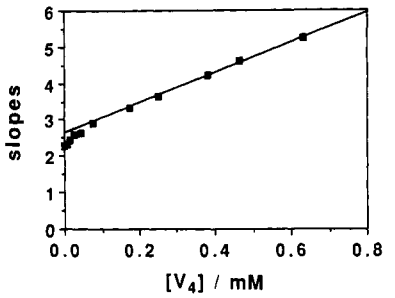

FIGURE 4: Lineweaver–Burk slopes are plotted as a function of vanadate tetramer concentrations. The linear dependence at high vanadate concentrations is indicated by a line.

The analysis discussed in the preceding paragraphs ignored the presumed formation of vanadyl cation when vanadate tetramer reacted with aldolase. The system at hand not only contains the inhibitory vanadate tetramer and dimer but also contains the inhibitory vanadyl cation. The complete inhibition scheme is

$$\begin{gathered} E + FDP \underset{k_{-s1}}{\overset{k_{s1}}{\rightleftarrows}} E{\cdot}FDP \xrightarrow{k_p} P \\ E + V(IV) \underset{k_{-1IV}}{\overset{k_{1IV}}{\rightleftarrows}} E{\cdot}V(IV) \\ E + V_2 \underset{k_{-12}}{\overset{k_{12}}{\rightleftarrows}} E{\cdot}V_2 \\ E + V_4 \underset{k_{-14}}{\overset{k_{14}}{\rightleftarrows}} E{\cdot}V_4 \xrightarrow{k_2} E^* \end{gathered} \tag{16}$$

The $K_{iV(IV)}$ is shown in eq 17, and the reciprocal rate is described by eq 18. The aldolase concentration is less than 0.2×10^{-6} M^{-1}, suggesting the V(IV) concentration will not exceed 10^{-6} M^{-1} significantly. The term $[V(IV)]/K_{iIV}$ in eq 18 will therefore be insignificant (if $[V(IV)] = 10^{-6}$ M^{-1}, then $[V(IV)]/k_{iIV} \approx 0.01$) despite the low K_i for V(IV). As a result, eq 18 simplifies to eq 15. No significant changes were made in the calculation of K_{iV2} and K_{iV4} when including V(IV) in the analysis.

$$K_{iV(IV)} = \frac{[E][V(IV)]}{[E{\cdot}V(IV)]} = \frac{k_{-1}}{K_1} \tag{17}$$

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[V_2]}{K_{iV2}} + \frac{[V(IV)]}{K_{iIV}} + \frac{K_{24}[V_2]^2}{K_{iV4}}\right) \tag{18}$$

Inhibition by Vanadate Decamer. The observed inhibition by oxoanions suggests that the more highly charged and the

more compact the anion, the better it will act as an inhibitor. The cyclic molybdates and vanadates are the most potent oxoanion inhibitors studied so far, suggesting the higher the charge and more compact the oxoanion, the better the inhibitor. Therefore, we examined the effects of a much larger derivative, the vanadate decamer ($V_{10}O_{28}^{6-}$), on aldolase. Inhibition measurements were carried out under the conditions described above, and it was assumed the decamer is a reversible inhibitor; the K_i was found to be 0.92 mM (or 9.2 mM vanadium atoms). Vanadate decamer forms at acidic pH, and ^{51}V NMR spectroscopy was used to ascertain that the stock solution indeed contained >98% vanadate decamer. The decamer hydrolyzes under the assay conditions to form the rapidly exchanging vanadate oligomers over the course of hours and days. To determine whether the observed inhibition was indeed caused by the decamer, EDTA was added to the assay solutions, complexing all of the rapidly exchanging vanadate oligomers. The addition of EDTA to the assay solutions did not affect the enzyme activity, demonstrating that inhibition is caused by the vanadate decamer and not by the other rapidly exchanging vanadate oligomers.

Surprisingly, the decamer showed a noncompetitive inhibition pattern, suggesting this anion is interacting differently with aldolase than the other oxoanions. The decamer is significantly larger than the vanadate tetramer and the cyclic molybdates described above. The largest distance from two oxygens (the major axis of the ellipsoid) was 9.9 Å, and the smallest distance (the minor axis of the ellipsoid) was 7.7 Å. These values compare to the largest diameter of 7.4 Å for the vanadate tetramer (Day et al., 1990) which because of the less dense structure could be squeezed to a pancake with a 3–4-Å width. It is possible that the decamer does not fit into the anion binding site and binds elsewhere on aldolase. Despite the large size of the decamer, it still shows higher affinity for aldolase than monomeric vanadate and other oxoanions. This comparison includes many strong organic phosphate inhibitors, such as ATP. The organic phosphate derived compounds lose their tight binding to aldolase at 0.4 M KCl (Table I). In our assay system, ATP had a K_i larger than 15 mM, whereas a low ionic strength this K_i is reported in the micromolar range (Palczewski et al., 1985). The low inhibition constant for the decamer at high salt concentrations may suggest such polyoxoanionic species will be extremely potent inhibitors at low ionic strengths.

Yonetani and Theorell Analysis of Oxoanion Inhibition of Aldolase. Initial rates were measured at fixed concentrations of FDP ($2K_m$) in the presence of two inhibitors simultaneously in order to analyze these according to the method described by Yonetani and Theorell (1964). The parallel lines of these experiments with vanadate and arsenate suggest the two oxoanions binds in a mutually exclusive manner. Analogous plots were found with vanadate and molybdate and with vanadate and tungstate. These studies suggest one binding site accommodates arsenate, molybdate, tungstate, and vanadate.

Similar studies with vanadate and phosphate yield plots that intersect, suggesting that the two anions are not mutually exclusive, although binding of one anion interferes with the binding of the other. The interaction constants for phosphate, pyrophosphate, and sulfate were 2.8, 2.8, and 1.3 mM, respectively. It is interesting that phosphate and pyrophosphate both have the same interaction constant with vanadate when inhibiting aldolase, and might suggest that phosphate and pyrophosphate bind in the same manner to aldolase whereas vanadate interacts very differently with the enzyme. The fact that a different interaction constant was observed for vanadate and sulfate suggests that neither vanadate nor sulfate binds to aldolase at the same site as phosphate. Vanadate and vanadium(IV) (vanadyl cation) interfere with the binding of each other; in this case, the interaction constant is 1.9 mM. It would be of interest to determine the location where vanadate binds to aldolase, and we therefore explored the possibility for using vanadate as a reporter group for anion binding.

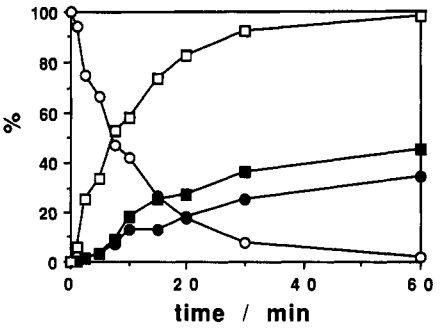

FIGURE 5: Photolysis experiment of vanadate-treated aldolase as a function of UV irradiation time (2.0 mM vanadate, 10 mM Tris, 5 mM $MgCl_2$, and 100 mM KCl, pH 7.5 and 4 °C). Percentage of enzyme activity (○); percentage of enzyme inhibition (■); percentage of total aldolase subunit cleavage (■) percentage of 36-kDa cleavage band (●).

Vanadate—A Potential Reporter Group for Aldolase. The use of UV light-induced oxidation of vanadate as a photocleavable agent for aldolase was explored. Aldolase at a concentration of 1 mg/mL was incubated with 2 mM vanadate in 0.10 and 0.40 M KCl, 5.0 mM $MgCl_2$, and 10 mM Tris at pH 7.1 and 8.0. Samples of aldolase with and without vanadate were irradiated from 0 to 60 min. Enzyme activity was measured before and after irradiation, and found to decrease after 60-min irradiation, thus limiting the treatment to 60 min. SDS–PAGE electrophoretic analysis revealed that polypeptide chain cleavage occurred in the presence of 2 mM vanadate, as shown by a major cleavage band at 36 kDa and two minor bands at 31 and 28 kDa. The cleavage of the aldolase subunit was quantified using densitometry, measuring the intensity of the emerging cleavage bands and the intensity of the original subunit at 41 kDa. Using the intensity of the cleavage bands, the percent cleavage could be estimated assuming the second cleavage band was too small to be retained on the PAGE gel. If, however, the gel was stopped before the dye front left the gel, a band at this front indicated that the low molecular weight bands were indeed too small to be retained by the gel. The appearance of total aldolase cleavage, the major cleavage bands, the enzyme activity, and the loss of enzyme activity were plotted as a function of irradiation time and shown in Figure 5. The correlation indeed suggests that the vanadate-induced photocleavage translates directly to loss of enzyme activity, although deactivation occurred and reduced the activity by 35% before irradiation took place. A plot of the total percent cleavage as a function of percent inhibition is shown in Figure 6. This initial phase of deactivation is presumably due to the irreversible inactivation by vanadate tetramer described earlier. At 2.0 mM vanadate, a 35% loss in activity by the tetramer is anticipated, and since aldolase and vanadate were incubated for 30 min before UV irradiation began, this inactivation complex presumably accounts for the initial loss in activity. These results show vanadate can be used as a reporter group for the anion binding site in aldolase despite the fact that vanadate tetramer already has irreversibly deactivated 35% of the enzyme. Further studies using vanadate as a probe including determination of

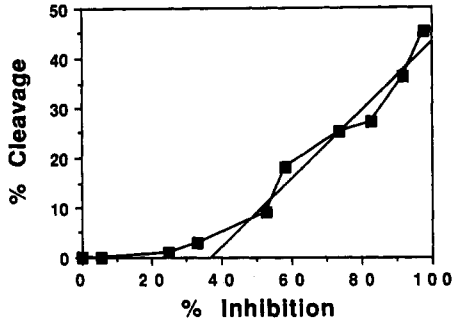

FIGURE 6: Percentage of vanadate-induced UV light cleavage is correlated with the percentage of enzyme activity of aldolase (2.0 mM vanadate, 10 mM Tris, 5 mM $MgCl_2$, and 100 mM KCl, pH 7.5 and 4 °C). At these conditions, 2.0 mM vanadate irreversibly modifies aldolase to the level of 35% inhibition.

the chemistry of the peptide cleavage and the locations of the cleavage sites by sequencing and mass spectrometry are ongoing.

CONCLUSION

Vanadate was used as a probe to explore the interactions of oxoanions with aldolase at ionic strengths approaching physiological levels. Solutions containing vanadate monomer, dimer, tetramer, and pentamer interact reductively, nonreductively, and photolytically with aldolase. The various types of competitive interactions were determined and the active vanadium derivatives identifed. Vanadate monomer does not interact significantly with aldolase. Vanadate dimer is a reversible inhibitor, and the K_{iV2} is 0.8 mM (or 1.6 mM vanadium atoms). Vanadate tetramer is both a reversible and an irreversible inhibitor of aldolase; the aldolase–tetramer complex which is initially formed can either dissociate or proceed to inactivate aldolase. The K_{iV4} is about 0.5 mM tetramer, corresponding to 2.0 mM vanadium atoms, and the rate constant for reduction of the aldolase–vanadate complex is 0.9 min^{-1} at 25 °C. In general, oxoanions inhibit aldolase much more weakly at high ionic strength, suggesting that anions such as ATP, inositol phosphates, and other potent inhibitors at low ionic strength will not significantly inhibit aldolase at physiological conditions. It appears that the higher the charges, and the more compact the anion, the more potent the inhibitor; we therefore examined the effects of vanadate decamer, which is a very large and compact oxoanion. Vanadate decamer was the only noncompetitive vanadium-based inhibitor of aldolase, which suggests that very large and compact anions may no longer interact competitively with aldolase. The interaction of aldolase is also sensitive to the nature of the anion, illustrated by the fact that arsenate is a better inhibitor than phosphate, but a weaker inhibitor that molybdate and tungstate. Yonetani–Theorell analysis suggests that some of these oxoanions bind to different sites on aldolase. Vanadate binds to a different site on aldolase than does phosphate, pyrophosphate, and sulfate. Phosphate and pyrophosphate probably bind to the same site, whereas sulfate binds differently. Arsenate, vanadate, tungstate, and molybdate all bind to the same site on aldolase; this site is different than the two sites phosphate or sulfate binds to. Vanadyl cation was found to be the most potent vanadium inhibitor with a K_i of 0.083 mM. Vanadate and vanadyl cation bind to different sites on aldolase. We explored the use of vanadate-induced photolytic cleavage of aldolase, despite the complex redox chemistry that occurs between vanadate tetramer and aldolase. Although inactivation of aldolase by vanadate tetramer occurred, a direct correlation between loss of enzyme activity and appearance of cleavage products was observed, suggesting vanadate will be a useful probe for future studies with aldolase.

ACKNOWLEDGMENT

We thank Dr. Robert W. Marshman for assistance in analyzing the data and reading the manuscript. We also thank Professor Michael T. Pope for bringing the cyclic molybdates to our attention.

REFERENCES

Barkigia, K. M., Rajkovic, L. M., Pope, M. T., & Quicksall, C. O. (1975) *J. Am. Chem. Soc. 97*, 4146–4147.
Boyd, D. W., Kustin, K., & Niwa, M. (1985) *Biochim. Biophys. Acta 827*, 472–475.
Chan, W. W.-C., Mort, J. S., Chong, D. K. K., & Macdonald, P. D. M. (1973) *J. Biol. Chem. 248*, 2778–2784.
Chasteen, N. D. (1981) in *Biological Magnetic Resonance* (Berliner, L., & Reuben, J., Eds.) Vol. 3, pp 53–119, Plenum, New York.
Cohen, M. D., Sen, A. C., & Wei, C.-I. (1987) *Inorg. Chim. Acta 138*, 179–186.
Crans, D. C., & Shin, P. K. (1988) *Inorg. Chem. 27*, 1797–1806.
Crans, D. C., & Schelble, S. M. (1990) *Biochemistry 29*, 6698–6706.
Crans, D. C., & Simone, C. M. (1991) *Biochemistry 30*, 6734–6741.
Crans, D. C., Rithner, C. D., & Theisen, L. A. (1990a) *J. Am. Chem. Soc. 112*, 2901–2908.
Crans, D. C., Willging, E. M., & Butler, S. R. (1990b) *J. Am. Chem. Soc. 112*, 427–432.
Cremo, C. R., Long, G. T., & Grammer, J. C. (1990) *Biochemistry 29*, 7982–7990.
Cremo, C. R., Grammer, J. C., & Yount, R. G. (1991) *Methods Enzymol. 196*, 442–449.
Csermely, P., Martnonosi, A., Levy, G. C., & Ejchart, A. J. (1985) *Biochem. J. 230*, 807–815.
Day, V. W., Klemperer, W. G., & Yagasaki, A. (1990) *Chem. Lett.*, 1267–1270.
DeMaster, E. G., & Mitchell, R. A. (1973) *Biochemistry 12*, 3616–3621.
Drueckhammer, D. G., Durrwachter, J. R., Pederson, R. L., Crans, D. C., Daniels, L., & Wong, C.-H. (1989) *J. Org. Chem. 54*, 70–77.
Erdmann, E., Krawietz, W., Philipp, G., Hackbarth, I., Schmitz, W., Scholz, H., & Crane, F. L. (1979) *Nature 282*, 335–336.
Gibbons, I. R., Lee-Eiford, A., Mocz, G., Phillipson, C. A., Tang, W.-J. Y., & Gibbons, B. H. (1987) *J. Biol. Chem. 262*, 2780–2786.
Ginsburg, A., & Mehler, A. H. (1966) *Biochemistry 5*, 2623–2634.
Grazi, E. (1975) *Biochem. J. 151*, 167–172.
Grazi, E., & Trombetta, G. (1987) *Eur. J. Biochem. 19*, 197–200.
Horecker, B. L., Tsolas, O., & Lai, C. Y. (1972) *Enzymes (3rd Ed.) 7*, 213–258.
Kasprzak, A. A., & Kochman, M. (1980) *Eur. J. Biochem. 104*, 443–450.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Kobashi, K., & Horecker, B. L. (1967) *Arch. Biochem. Biophys. 121*, 178–186.
Koppitz, B., Vogel, F., & Mayr, G. W. (1986) *Eur. J. Biochem. 161*, 421–433.
Kustin, K., & Toppen, D. L. (1973) *Inorg. Chem. 12*, 1404–1407.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Matteuzzi, M., Bellini, T., Bergamini, C. M., & Dallocchio, F. (1985) *Biochem. Int. 10*, 53–62.
Mehler, A. H. (1963) *J. Biol. Chem. 238*, 100–104.

Newman, L., LaFleur, W. J., Brousaicles, F. J., & Ross, A. M. (1958) *J. Am. Chem. Soc. 80*, 4491–4495.

Offermann, M. K., McKay, M. J., Marsh, M. W., & Bond, J. S. (1984) *J. Biol. Chem. 259*, 8886–8891.

Palczewski, K., & Kochman, M. (1987) *Biochemistry 26*, 3466–3471.

Palczewski, K., Hargrave, P. A., Folta, E. J., & Kochman, M. (1985) *Eur. J. Biochem. 146*, 309–314.

Pettersson, L., Andersson, I., & Hedman, B. (1985) *Chem. Scr. 25*, 309–317.

Pope, M. T. (1983) *Heteropoly and Isopoly Oxometalates*, Springer-Verlag, New York.

Ramasarma, T., MacKellar, W. C., & Crane, F. L. (1981) *Biochim. Biophys. Acta 646*, 88–98.

Rose, I. A., & O'Connell, E. L. (1969) *J. Biol. Chem. 244*, 126–134.

Rose, I. A., & Warms, J. V. B. (1985) *Biochemistry 24*, 3952–3957.

Sanchez de Jimenez, E., Lee, E., Torres, J., & Soberon, G. (1964) *J. Biol. Chem. 239*, 4154–4158.

Segel, I. H. (1969) *Enzyme Kinetics*, John Wiley & Sons, New York.

Soman, G., Chang, Y. C., & Graves, D. J. (1983) *Biochemistry 22*, 4994–5000.

Spolter, P. D., Adelman, R. C., & Weinhouse, S. (1965) *J. Biol. Chem. 240*, 1327–1337.

Stalick, J. K., & Quicksall, C. O. (1976) *Inorg. Chem. 15*, 1577–1584.

Sygusch, J., Beaudry, D., & Allaire, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7846–7850.

Vyskocil, F., Teisinger, J., & 'DLouha, H. (1980) *Nature (London) 286*, 516–517.

Yonetani, T., & Theorell, H. (1964) *Arch. Biochem. Biophys. 106*, 243–251.

Registry No. V_{i2}, 22466-30-4; V_{i4}, 78197-79-2; Zn, 7440-66-6; $[(CH_3)_2AsMo_4O_{14}OH]^{2-}$, 56652-66-5; $[(C_6H_5P)_2Mo_5O_{21}]^{5-}$, 57284-27-2; aldolase, 9024-52-6; phosphate, 14265-44-2; pyrophosphate, 14000-31-8; sulfate, 14808-79-8; arsenate, 15584-04-0; molybdate, 14259-85-9; tungstate, 14311-52-5; fructose 6-phosphate, 643-13-0; V decamer, 12397-12-5; vanadyl cation, 20644-97-7; fructose bisphosphate, 488-69-7.