α-Fluoro Acid and α-Fluoro Amide Analogs of Acetyl-CoA as Inhibitors of Citrate Synthase: Effect of pK_a Matching on Binding Affinity and Hydrogen Bond Length[†,‡]

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ABSTRACT: An α-fluoro acid analog and an α-fluoro amide analog of acetyl-CoA have been synthesized. The ternary complexes of these inhibitors with oxaloacetate and citrate synthase have been crystallized and their structures analyzed at 1.7 Å resolution. The structures are similar to those reported for the corresponding non-fluorinated analogs (Usher et al., 1994), with all forming unusually short hydrogen bonds to Asp 375. The α-fluoro amide analog binds with an affinity 1.5-fold lower than that of a previously described amide analog lacking the α-fluoro group. The α-fluoro acid analog binds with a 50-fold decreased affinity relative to the corresponding unfluorinated analog. The binding affinities are consistent with increased strengths of hydrogen bonds to Asp 375 with closer matching of pK_a values between hydrogen bond donors and acceptors. The results do not support any direct correlation between hydrogen bond strength and hydrogen bond length in enzyme−inhibitor complexes.

We recently reported the presence of unusually short hydrogen bonds in the structures of the complexes of citrate synthase with carboxylate and amide analogs of acetyl-CoA[1] (Usher et al., 1994). Short strong hydrogen bonds have recently been proposed to play a major role in the stabilization of enzyme-bound intermediates and/or transition states (Gerlt & Gassman, 1993a,b; Cleland & Kreevoy, 1994). In small molecules, very short hydrogen bonds are often observed between hydrogen bond donor and acceptor having matched pK_a's, so that the proton is equally shared in a symmetrical, single potential energy well hydrogen bond (Perrin, 1994). Such hydrogen bonds, also called "low barrier" hydrogen bonds, have measured gas phase energies of 30 kcal/mol or greater, compared to about 8 kcal/mol for normal, asymmetric hydrogen bonds (Hibbert & Emsley, 1990). Several examples of low barrier hydrogen bonds in enzyme complexes have now been reported, based on X-ray crystal structure and NMR evidence (Fraser et al., 1992; Frey et al., 1994; Tong & Davis, 1995; Tronrud et al., 1987; Usher et al., 1994; Xiang et al., 1995).

The proposed role of short, strong hydrogen bonds in enzyme catalysis is based on the formation of an extremely strong pK_a-matched hydrogen bond in an enzyme−intermediate or enzyme−transition state complex from a much weaker pK_a-mismatched hydrogen bond in the enzyme−substrate complex. In order to better analyze the effect of pK_a matching on strengths of hydrogen bonds in enzyme−inhibitor complexes, we have now prepared the α-fluoro analogs of the previously studied carboxylate and amide inhibitors. We report here the synthesis of these analogs, studies of their inhibition of citrate synthase, and the structures of the citrate synthase−inhibitor complexes. The results are consistent with moderately increased strengths of hydrogen bonds with closer matching of pK_a values between hydrogen bond donors and acceptors. However, the results do not support a direct correlation between hydrogen bond strength and hydrogen bond length in enzyme−inhibitor complexes.

MATERIALS AND METHODS

Synthesis of Acetyl-CoA Analogs FCMX and FAMX. (A) α-Fluoro-γ-butyrolactone. To a solution of α-hydoxy-γ-butyrolactone (5 g, 49.0 mmol) in 300 mL of dry methylene chloride at 0 °C was slowly added diethylaminosulfur trifluoride (DAST) (7.0 mL, 53.0 mmol). The reaction was allowed to warm to room temperature and was quenched after 20 h with the addition of iced, saturated aqueous sodium bicarbonate (200 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (3 × 100 mL), and the combined aqueous layers were extracted with methylene chloride (3 × 100 mL). The organic layers were combined, dried over magnesium sulfate, and concentrated in vacuo to yield α-fluoro-γ-butyrolactone as a yellow oil (5.05 g, 48.5 mmol): ^{1}H-NMR ($CDCl_3$) δ 2.4−2.8 (m, 2 H), 4.25−4.6 (m, 2 H), 5.2 (dt, 1 H, J_{H-H} = 8.0 Hz, J_{H-F} = 51.3 Hz);

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[‡] The atomic coordinates for FAMX and FCMX have been deposited in the Brookhaven Protein Data Bank under Accession Numbers 1CSR and 1CSS, respectively.

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[1] Abbreviations: CoA, coenzyme A; rms, root mean square; FCMX, α-fluorocarboxymethyldethia coenzyme A; FAMX, α-fluoroamidocarboxymethyldethia coenzyme A; CMX, carboxymethyldethia coenzyme A; AMX, amidocarboxymethyldethia coenzyme A; CMC, carboxymethyl coenzyme A.

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^{13}C-NMR δ 28.93 (d, J_{C-F} = 79.0 Hz), 64.74 (d, J_{C-F} = 25.0 Hz), 85.23 (d, J_{C-F} = 750.0 Hz), 171.99 (d, J_{C-F} = 84.2 Hz).

(B) 2-Fluoro-4-hydroxybutanamide. α-Fluoro-γ-butyrolactone (3.65 g, 35.1 mmol) was placed in a 500 mL 3-neck flask equipped with a stir bar. The flask was cooled to −78 °C with a dry ice/acetone bath, and a cold trap was placed on the middle neck and filled with a dry ice/acetone slurry. Ammonia (100 mL) was added to the reaction slowly. The reaction was stirred at −78 °C for 1 h and then allowed to slowly warm to room temperature, yielding 2-fluoro-4-hydroxybutanamide as a yellow solid (3.91 g, 32.3 mmol): ^{1}H-NMR ($CDCl_3$) δ 2.0−2.4 (m, 2 H), 3.85 (t, 2 H, J_{H-H} = 4.5 Hz), 5.1 (ddd, 1 H, J_{H-H} = 3.9 Hz, 6.0 Hz, J_{H-F} = 40.6 Hz), 5.7−5.9 (s, 1H), 6.3−6.5 (s, 1 H).

(C) 2-Fluoro-4-toluenesulfonyloxybutanamide. To a dry solution of 2-fluoro-4-hydroxybutanamide (3.91 g, 32.3 mmol) in pyridine (100 mL, 4 equiv) cooled to 5 °C was slowly added (10 min) toluenesulfonyl chloride (6.77 g, 35.5 mmol). After 3 h at 5 °C, the reaction was quenched with 100 mL of 4 M HCl. The aqueous layer was extracted with ether (3 × 100 mL), and the organic layers were then combined and washed with 0.5 M HCl (2 × 50 mL), dried over magnesium sulfate, and concentrated in vacuo to yield 2-fluoro-4-toluenesulfonyloxybutanamide (4.88 g, 23.1 mmol) as a yellow oil: ^{1}H-NMR ($CDCl_3$) δ 2.0−2.6 (m, 2 H), 2.5 (s, 3 H), 4.2 (d, 1 H, J_{H-H} = 5.2 Hz), 4.22 (d, 1 H, J_{H-H} = 5.2 Hz), 4.96 (ddd, 1 H, J_{H-H} = 4.0 Hz, 8.8 Hz, J_{H-F} = 49.6 Hz), 5.9−6.0 (s, 1 H), 6.3−6.4 (s, 1 H), 7.36 (d, 2 H, J_{H-H} = 8.4 Hz), 7.80 (d, 2 H, J_{H-H} = 8.6 Hz); ^{13}C-NMR δ 21.58 (s), 31.57 (d, J_{C-F} = 80 Hz), 65.26 (d, J_{C-F} = 14.2 Hz), 87.63 (d, J_{C-F} = 747.4 Hz), 127.81 (s), 129.88 (s), 132.41 (s), 145.05 (s), 171.7 (d, J_{C-F} = 80.6 Hz).

(D) 4-Azido-2-fluorobutanamide. To a solution of 2-fluoro-4-toluenesulfonyloxybutanamide (4.73 g, 22.4 mmol) in dimethyl sulfoxide (60 mL) was added sodium azide (2.91 g, 44.8 mmol). The reaction was heated under nitrogen at 50 °C for 12 h. Water was then added, and the solution was extracted with ether (3 × 100 mL). The organic layers were combined and washed with water (100 mL), dried over magnesium sulfate, and concentrated in vacuo to yield a yellow solid. The solid was further purified on silica gel (200 mL) with ethyl acetate (R_f = 0.38, visualized with iodine). The fractions containing product were concentrated in vacuo to yield 4-azido-2-fluorobutanamide (0.98 g, 7.7 mmol) as clear, spindly crystals: ^{1}H-NMR ($CDCl_3$) δ 2.0−2.5 (m, 2H), 3.5 (dt, 2 H, J_{H-H} = 7.4 Hz, J_{H-F} = 2.0 Hz), 5.0 (ddd, 1 H, J_{H-H} = 3.8 Hz, 8.2 Hz, J_{H-F} = 49.4 Hz), 6.2−6.4 (s, 1 H), 6.3−6.5 (s, 1 H), ^{13}C-NMR δ 31.47 (d, J_{C-F} = 80 Hz), 46.59 (d, J_{H-F} = 14.4 Hz), 88.72 (J_{H-F} = 744.4 Hz), 172.42 (d, J_{H-F} = 80.2 Hz).

(E) 4-Amino-2-fluorobutanamide. To a solution of 4-azido-2-fluorobutanamide (0.86 g, 6.77 mmol) in tetrahydrofuran (25 mL) was added triphenylphosphine (1.77 g, 6.77 mmol) and water (146.2 μL, 8.12 mmol). The reaction was allowed to stir at room temperature for 18 h. THF was removed in vacuo, and the remaining residue was redissolved in 75 mL of methylene chloride. The organic layer was washed with water (2 × 25 mL). The aqueous layers were combined and concentrated in vacuo to yield 4-amino-2-fluorobutanamide (0.73 g, 6.08 mmol) as a white solid which was used without further purification in the CoA coupling reaction: ^{1}H-NMR (D_2O) δ 1.8−2.1 (m, 2 H), 2.7 (dt, 2 H, J_{H-H} = 7.0 Hz, J_{H-F} = 1.4 Hz), 5.0 (ddd, 1 H, J_{H-H} = 4.2 Hz, 7.4 Hz, J_{H-F} = 48.8 Hz); ^{13}C-NMR δ 37.09 (d, J_{C-F} = 79.8 Hz), 38.86 (d, J_{C-F} = 13.2 Hz), 92.18 (d, J_{C-F} = 726.0 Hz), 177.98 (d, J_{C-F} = 83.2 Hz).

(F) α-Fluorocarboxymethyldethio Coenzyme A (FCMX). 4-amino-2-fluorobutyric acid (Hoshi et al., 1990) (2 mmol) was dissloved in 1 mL of water, and the pH was adjusted to 10.0 with 4 M NaOH. Twenty milligrams mg of adenosine 5′-(trihydrogen diphosphate) 3′-(dihydrogen phosphate) 5′-[(*R*)-3-hydroxy-4-[[3-(propylthio)-3-oxopropyl]amino]-2,2-dimethyl-4-oxobutyl] ester (Martin et al., 1994) was added, and the pH was again adjusted to 10.0. The reaction was followed by analytical reverse-phase HPLC using conditions previously described (Martin et al., 1994). The product had a retention time of 10.1 min, and the side-product due to hydrolysis of the thioester had a retention time of 10.7 min. After 28 h, all of the thioester starting material (retention time = 16.8 min) was gone, and the reaction was quenched by adjusting the pH to 4 and lyophilizing to yield a white powder (80% product by HPLC). The reaction was purified by preparative reverse-phase HPLC, and residual phosphates were removed using a SPICE cartridge: calcd MW = 810.1314, found = 810.1348; ^{1}H-NMR (D_2O) δ 0.65 (s, 3 H), 0.77 (s, 3 H), 1.8−2.0 (m, 2 H), 2.34 (t, 2 H, J_{H-H} = 6.0 Hz), 3.1−3.4 (m, 2 H), 3.35 (t, 2 H, J_{H-H} = 5.6 Hz), 3.58 (dd, 2 H, J_{H-H} = 5.2 Hz, 104.8 Hz), 3.89 (s, 1 H), 4.14 (s, 2 H), 6.10 (d, 2 H, J_{H-H} = 6 Hz), 8.24 (s, 1 H), 8.49 (s, 1 H).

(G) α-Fluorocarbamoylmethyldethio Coenzyme A (FAMX). 4-Amino-2-fluorobutanamide (2 mmol) was dissolved in 1 mL of water, and the pH was adjusted to 10.2 with 4 M HCl. Thirty milligrams mg of adenosine 5′-(trihydrogen diphosphate) 3′-(dihydrogen phosphate) 5′-[(*R*)-3-hydroxy-4-[[3-(propylthio)-3-oxopropyl]amino]-2,2-dimethyl-4-oxobutyl] ester (Martin et al., 1994) was added, and the pH was again adjusted to 10.2 using 4 M NaOH. The reaction was monitored by analytical reverse-phase HPLC. The product had a retention time of 10.7 min versus 9.7 min for the hydrolysis side product. After 7 h, complete disapperance of starting material was observed, and the reaction was quenched by adjusting the pH to 4 and lyophilizing to yield a white powder (90% product by HPLC). Purification was performed as for the α-fluorocarboxylate analog: calcd MW = 808.1395, found = 808.1378; ^{1}H-NMR (D_2O) δ 0.78 (s, 3 H), 0.91 (s, 3 H), 1.9−2.1 (m, 2 H), 2.43 (t, 2 H, J_{H-H} = 6.4 Hz), 3.29 (t, 2 H, J_{H-H} = 6.0 Hz), 3.44 (t, 2 H, J_{H-H} = 6.8 Hz), 3.70 (ddd, 2 H, J_{H-H} = 4.8 Hz, 9.6 Hz, 103.2 Hz), 3.99 (s, 1 H), 4.98 (ddd, 1 H, J_{H-H} = 4.4 Hz, 8 Hz, 48.4 Hz), 6.20 (d, 1 H, J_{H-H} = 3.6 Hz), 8.41 (s, 1 H), 8.65 (s, 1 H).

X-ray Crystallographic Studies of Citrate Synthase. Ternary complexes of chicken heart citrate synthase (Sigma), oxaloacetate, and FAMX or FCMX inhibitors were cocrystallized in a "closed" conformation crystal form identical to that seen in several previous studies (Remington et al., 1982; Usher et al., 1994). The enzyme (0.2 mM in 0.5 M citrate, pH 6.0) was mixed with inhibitor (2 mM) and oxaloacetate (5 mM) before crystallization by vapor diffusion over sodium citrate (0.9−1.1 M, pH 6.0). Crystals grew in space group *C2* with cell dimensions a = 104.4 Å, b = 78.5 Å, c = 58.5 Å, and β = 78.9° with external dimensions up to 0.4 × 0.3 × 0.2 mm. Data from a single FCMX crystal were collected using a Xuong−Hamlin multiwire detector and processed

using the supplied software (Howard et al., 1985). Data from a single FAMX crystal were collected and processed using an R-Axis image plate system and accompanying software from Molecular Structures Corp. Initial atomic coordinates in each case were taken from the final refined model of the previously solved structure of citrate synthase complexed with the AMX CoA analogue and oxaloacetate (Usher et al., 1994), excluding the terminal seven atoms of the inhibitor, the active site side chains His 274 and Asp 375, and two bound water molecules in the active site. Coordinates were refined using the TNT program package (Tronrud et al., 1987). After initial coordinate refinement had converged, the missing atoms were modeled in on the basis of the $F_o - F_c$ difference electron density map, along with the new fluorine atom of the inhibitor. In the case of FCMX, where the density for two different epimers of the fluorine atom was visible, a double-headed model of the inhibitor, containing duplicate copies of its terminal six atoms, was used in order to assess possible differences between the two epimers of the inhibitor. These atoms were set with occupancies of 0.5 each and were initially placed in close to overlapping positions (except for the two distinct fluorine positions), and bad contact restraints between the two copies were turned off. After this model-building using the FRODO programs (Jones, 1978), further coordinate refinement was done, followed by combined coordinate/correlated temperature factor refinement. Analysis of the structures was based on these final models, as well as on $F_o - F_o$ difference maps calculated between scaled diffraction data sets from the inhibitor complexes in this study and from the previously published AMX and CMX inhibitor structures (Usher et al., 1994).

Inhibition Studies of Citrate Synthase. For inhibition studies of citrate synthase with the acetyl-CoA analogs, assays were conducted as described previously (Srere, 1961) in 0.1 M Tris, pH 8, containing 0.4 mM oxaloacetate, 0.005–0.09 mM acetyl-CoA, and 0.037 unit of citrate synthase (from porcine heart). The reactions were monitored at 233 nm using $\epsilon_{233} = 5.4 \times 10^3\ M^{-1}\ cm^{-1}$ for the thioester. K_i values were calculated from double-reciprocal plots of $1/v$ vs 1/[acetylCoA] at four concentrations of the inhibitors.

RESULTS

The α-fluoro acid (FCMX) and α-fluoro amide (FAMX) analogs of acetyl-CoA (Figure 1) were prepared by aminolysis reactions of the thioester bond of a versatile CoA analog synthon as described under Materials and Methods and following general procedures reported previously (Martin et al., 1994). FCMX and FAMX differ from the previously studied CMX and AMX (Martin et al., 1994; Usher et al., 1994) only in that one of the α-hydrogens is replaced with a fluorine atom.

The crystal structures of the ternary complexes of citrate synthase and oxaloacetate with FCMX and FAMX have been solved. Refined atomic coordinates have been deposited with the Brookhaven Protein Data Bank, Accession Numbers 1CSR (FAMX) and 1CSS (FCMX). Both analogs bind as expected based on structures of the CMX and AMX complexes and the proposed binding mode of acetyl-CoA. The carboxylate and amide functional groups interact with His 274 and Asp 375 (Figure 2). The F(o,FAMX) − F(o,AMX) difference map (Figure 3A) has only one significant feature: additional electron density contributed by the fluorine atom attached to the α-carbon of FAMX. The lack of other features demonstrates that FAMX binding is indeed essentially identical to that of AMX. Accordingly, the measured Asp 375/inhibitor hydrogen bonding distance of 2.51 Å matches that published for AMX (2.49 ± 0.02 Å). While FAMX was synthesized as a mixture of epimers at the α-fluoro position, the enzyme selectively binds only the *R* stereoisomer, presumably because binding of the other stereoisomer is disfavored by a close contact with the side chains of Asn 373 and Asp 375. The F(o,FAMX) − F(o,CMX) difference map (data not shown) shows the same set of differences in the critical Asp 375/inhibitor interaction as were seen previously with the F(o,AMX) − F(o,CMX) map (Usher et al., 1994).

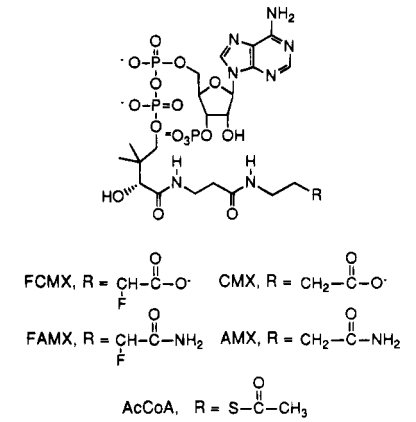

FIGURE 1: Covalent structures of inhibitors used (α-fluoro carboxylate, FCMX; and α-fluoro primary amide, FAMX) and referred to (carboxylate, CMX; and primary amide, AMX) in this work, and of acetyl-CoA, the substrate used by citrate synthase.

Table 1: Binding Constants (pH 8.0) and Estimated pK_a Values for Key Functional Groups in Citrate Synthase–Inhibitor Complexes

compound	K_i (or K_m) (M)	estimated pK_a
acetyl-CoA	1.6×10^{-5} (K_m)	
CMX	1.6×10^{-8}	3 (anti)
AMX	2.8×10^{-8}	15
FCMX	8.0×10^{-7}	1 (anti)
FAMX	4.2×10^{-8}	12
Asp 375		5–6 (syn)

While only one stereoisomer of FAMX is observed in the structure of the enzyme complex, the two stereoisomers of FCMX appear to bind in near equal occupancy. Modeling and refinement of the coordinate models of the mixture of both α-fluoro epimers are subject to some uncertainty, and show only that the distance is in the range from 2.3 to 2.6 Å. However, direct comparison of $F_o - F_o$ difference maps shows convincingly that the interactions of the fluorocarboxylate are identical to those of CMX, for which the Asp 375/inhibitor hydrogen bond length has been previously determined as 2.4 Å [see Usher et al. (1994) for a discussion of the accuracy of this measurement.] As with the amide analogs, the F(o,FCMX) − F(o,CMX) difference map (Figure 3B) shows only the additional electron density contributed by the fluorine atom attached to the α-carbon of FCMX. In contrast, the F(o,FCMX) − F(o,AMX) difference map (Figure 4A) shows difference features around the carboxylate of Asp 375 and the inhibitor next to it that correspond closely to those seen previously in the F(o,CMX) − F(o,AMX) map (Figure 4B). These features are a result

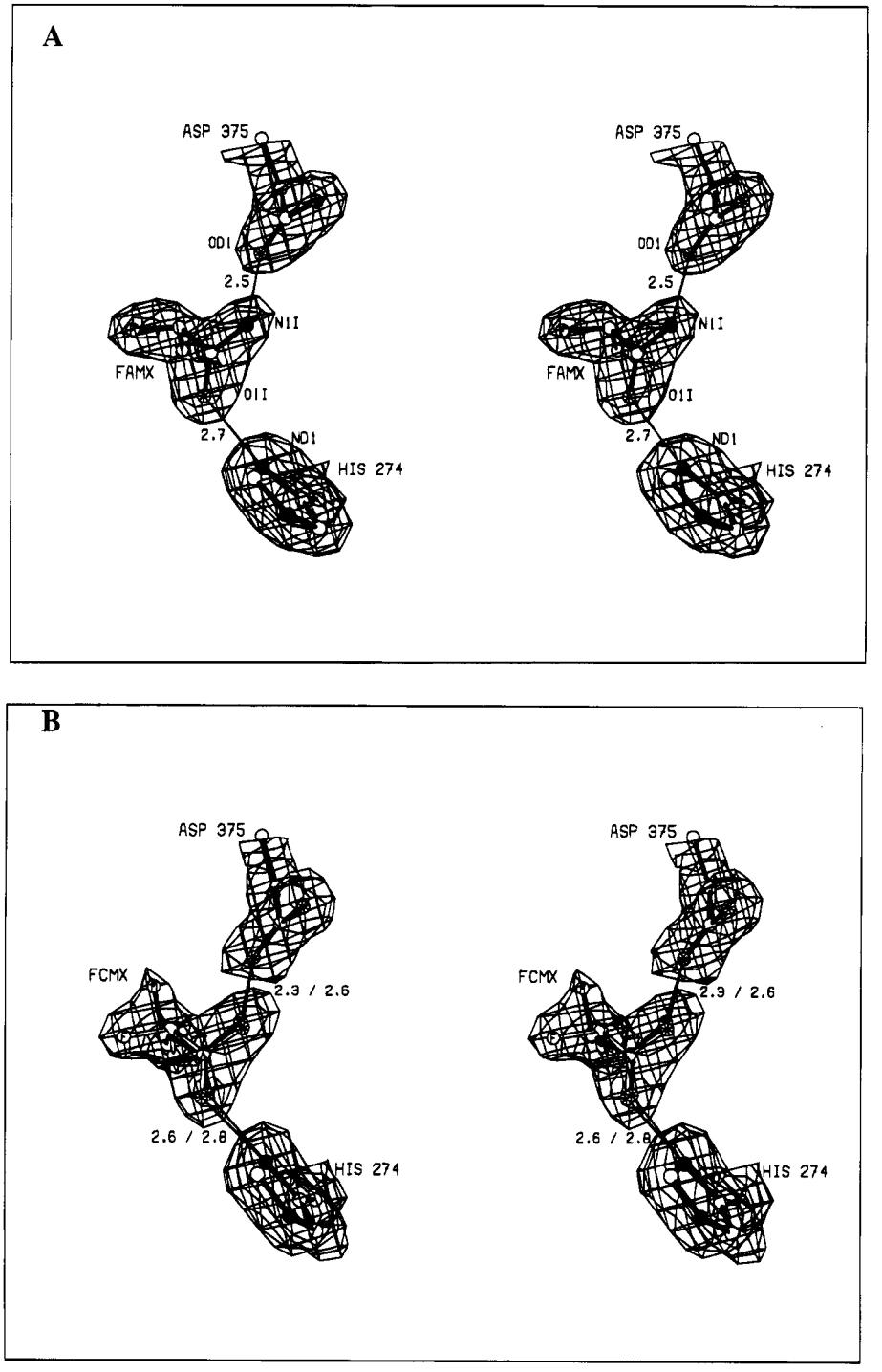

FIGURE 2: Stereoview of the final models and $2F_o - F_c$ electron density maps contoured at 1.5 standard deviations. Carbon atoms are open, nitrogen atoms are filled, oxygen atoms are spoked, and fluorine atoms are labeled by F. (A) ternary complex with FAMX (a-fluoro primary amide analogue), showing the single position of the α-fluoro atom in the *R* stereoisomer, as well as the hydrogen bond to Asp-375. (B) FCMX (α-fluorocarboxyl analogue), showing an averaged model with fluorine atttached in both *R* and *S* configurations.

of a slight shortening of the hydrogen bond with either carboxylate bound, as opposed to AMX.

FCMX and FAMX were tested as inhibitors of citrate synthase at pH 8.0. The results, along with previously reported results for the nonfluorinated analogs CMX and AMX and estimated pK_a values for groups that form hydrogen bonds to Asp375 are shown in Table 1. FAMX was found to be a less potent inhibitor than AMX by a factor of 1.5, while FCMX had an approximately 50-fold decrease in affinity relative to CMX.

DISCUSSION

In previous work, a carboxylate oxygen of CMX was found to form a very short hydrogen bond to a carboxylate oxygen of Asp 375 in a complex with citrate synthase (Usher et al., 1994). It was inferred that these two groups have sufficiently matched pK_a values to form a symmetrical or low barrier hydrogen bond, despite the presumed difference in pK_a values of syn vs anti carboxylates (Gandour, 1981; Gandour et al., 1990). AMX was found to form a slightly longer, though still unusually short, hydrogen bond to Asp

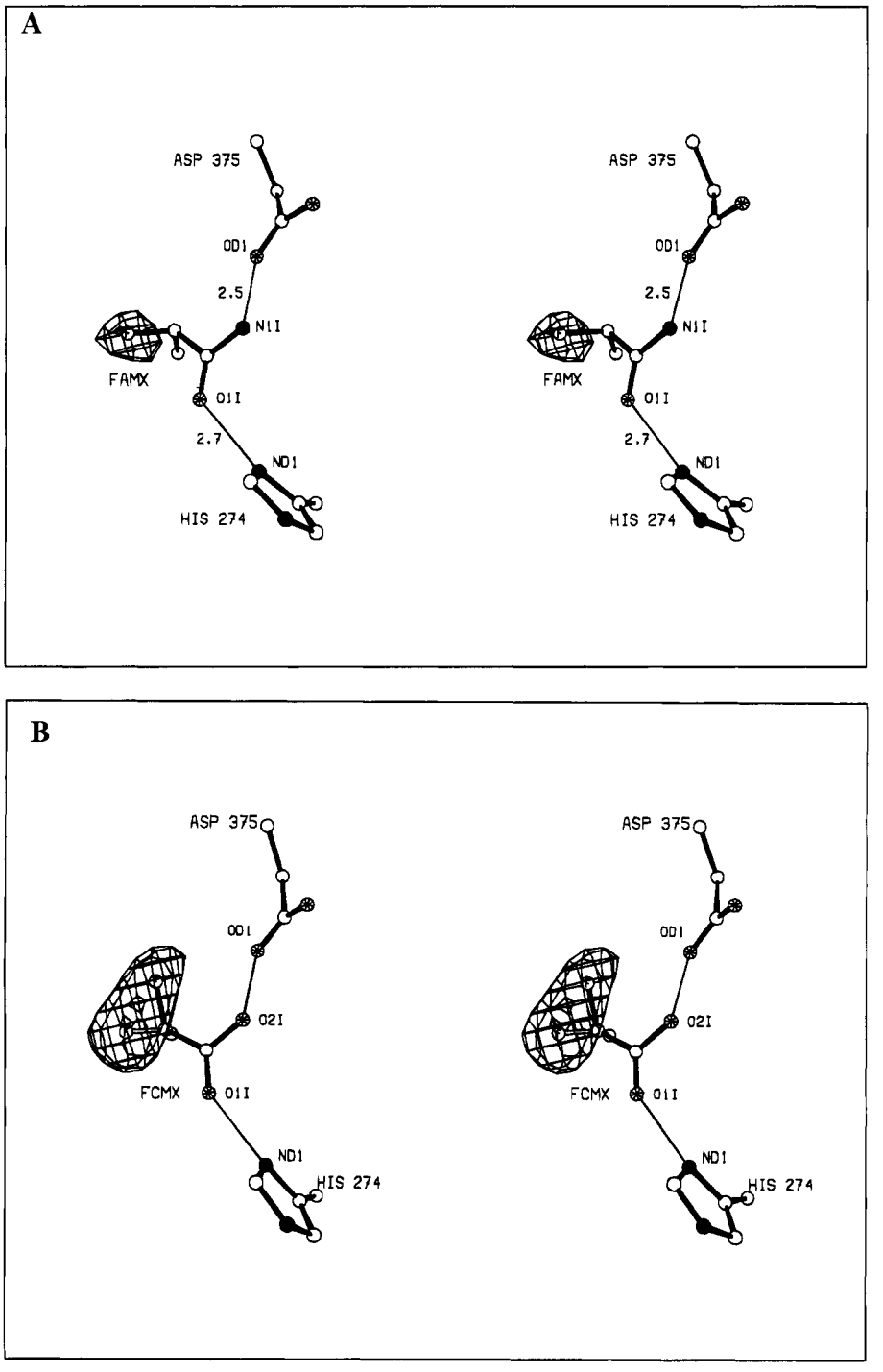

FIGURE 3: $F_o - F_o$ difference maps, contoured at ±4 standard deviations. (A) F(o,FAMX) − F(o,AMX) with the final refined position of the FAMX model shown. The single positive peak at the position of the R-fluoro replacement is the only significant feature of the map. (B) F(o,FCMX) − F(o,CMX) with two overlapping models shown, representing the R (open bonds) and S (filled bonds) stereoisomers. A double positive peak at the positions of the R/S-fluoro replacement is the only significant feature of the map.

375, despite the large pK_a mismatch. Comparison of binding affinities of CMX and AMX suggested a relatively small difference in the strengths of the short hydrogen bonds between groups with near-matched and very mismatched pK_a values. FCMX was prepared in order to further probe the effect of pK_a matching on the strengths of hydrogen bonds to Asp 375.

Fluoroacetic acid has a pK_a of 2.6, compared to a pK_a of 4.7 for acetic acid. A similar lowering of the pK_a by about 2 units is assumed for the acetyl-CoA analog. The relative binding of FCMX vs CMX could also be influenced by effects of the fluorine other than its altering of the carboxylate pK_a. Being larger than hydrogen, the fluorine atom could cause unfavorable steric interactions with amino acids in the active site. The fluorine can also form hydrogen bonds with solvent water, which could influence its partitioning between freely solvated and enzyme-bound states. To help analyze the various effects of fluorine on binding, FAMX was prepared. Comparison of FAMX binding with that of AMX should provide insights into effects other than the pK_a of

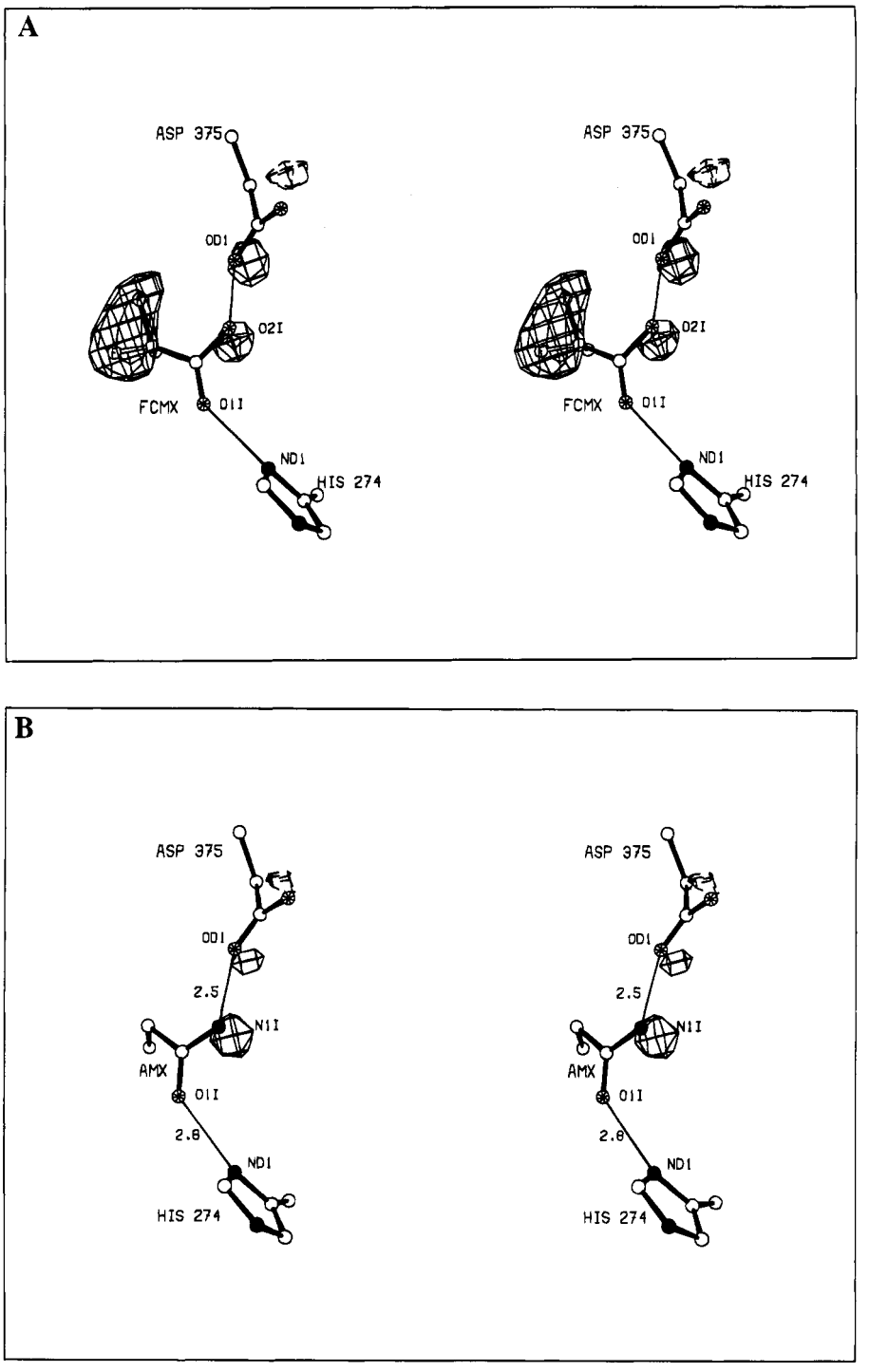

FIGURE 4: $F_o - F_o$ difference maps, contoured at ±4 standard deviations. (A) F(o,FCMX) − F(o,AMX) with the final refined position of the FCMX model shown. In addition to the fluorine peaks, features around O2I of FCMX and OD1/OD2 of Asp 375 indicate that these groups move together in the FCMX structure. (B) F(o,CMX) − F(o,AMX) with the final refined position of the AMX model shown. Features around N1I of AMX and OD1/OD2 of Asp 375 indicate that these groups move apart in the AMX structure.

the fluorine substitution on citrate synthase binding.

The structures of the FCMX and FAMX complexes of citrate synthase support their design as probes for comparison of hydrogen bond strengths with those in the CMX and AMX complexes. All four inhibitors bind very similarly, with only slight positional adjustment between the acid vs amide analogs. One unexpected result is that citrate synthase binds a single stereoisomer of FAMX but binds both stereoisomers of FCMX in near-equal occupancy. This indicates that the binding of the amide and the binding of the carboxylate are not quite identical. This, however, is not entirely surprising: while both inhibitors have the same number of non-hydrogen atoms and both form the same number of hydrogen bonds with citrate synthase, FCMX acts as an acceptor of hydrogen bonds where FAMX is a donor. This slight change is sufficient to prevent FAMX from binding in both modes, but is not a major factor otherwise. Most importantly, it does not substantially affect the conclusions drawn from the

Table 2: Diffraction Data and Atomic Model Statistics

	data set	
	FAMX	FCMX
Refinement Statistics		
observations	67827	77967
unique reflections	38748	44220
resolution (Å)	1.70	1.70
completeness (%)[a] (overall)	75	86
25−2.7 Å (%)	96	96
2.7−2.0 Å (%)	86	91
2.0−1.7 Å (%)	52	74
R-merge (%)[b]	7.7	4.4
R-factor (%)[c]	17.9	16.5
no. of atoms in model		
protein	3391	3391
ligands	61	61
solvent	145	145
Deviations from Ideality (rms)		
bond lengths (Å)	0.012	0.014
bond angles (deg)	2.5	2.8
bad contacts (Å)	0.018	0.018
B correlations (Å^2)	5.2	5.0

[a] Completeness is the ratio in percent of observed to theoretically possible reflections. [b] *R*-merge gives the average disagreement in percent for repeated measurements of an intensity. [c] *R*-factor is the standard crystallographic reliability factor.

relative binding affinities of the inhibitors.

An interesting feature of the X-ray structural data is the relative hydrogen bond lengths between Asp 375 and the carboxylate oxygens of CMX and FCMX, based on the *F*(o,FCMX) − *F*(o,CMX) difference map. While there is some degree of uncertainty in the absolute bond lengths, the data show with confidence that the hydrogen bonds formed by FCMX and CMX to Asp 375 are essentially identical in length, both being very short. A difference in bond length as small as 0.1 Å would lead to substantial density in the difference map, as observed in the *F*(o,FCMX) − *F*(o,AMX) and *F*(o,CMX) − *F*(o,AMX) difference maps. Likewise, the *F*(o,FAMX) − *F*(o,AMX) difference map shows no difference in lengths of the hydrogen bonds with Asp 375 between these two inhibitors.

While the difference in hydrogen bond lengths to Asp 375 between the carboxylate and carboxamide inhibitors is readily demonstrable, it is not large, and indeed the interactions of all of the inhibitors with Asp 375 are quite unusually short. This suggests that they may all be forming the same kind of interaction, possibly a low-barrier hydrogen bond. It is quite possible that the small distance increase seen when AMX binds is simply due to substitution of nitrogen vs oxygen as a donor/acceptor. Indeed, a slight (0.03 Å) increase is expected simply on the basis of the van der Waals radii of N (1.55 Å) versus O (1.52 Å) (Hibbert & Emsley, 1990). Analysis of hydrogen bonding in globular proteins shows a mean NH−O distance of 2.95 Å, slightly longer than the mean OH−O distance of 2.86 Å (Baker & Hubbard, 1984). If this does account for the small difference observed, then pK_a matching is not an important part of the interaction.

It is believed that AMX and FAMX bind with the amide nitrogen forming a hydrogen bond to Asp 375, with the amide carbonyl oxygen hydrogen bonding to His 274 and water, based on analysis of the *F*(o,CMX) − *F*(o,AMX) difference map (Usher et al., 1994). The various pK_a values involved in hydrogen bonding to Asp 375 can be estimated as shown in Table 1. The pK_a values for the anti orbitals of CMX and FCMX are based on known pK_a values for acetic and fluoroacetic acids and conservative estimates of pK_a differences between syn and anti orbitals (Gandour, 1981; Gandour et al., 1990). The predicted value for FAMX is based on extrapolation between acetamide (pK_a = 15) (Bordwell, 1988) and trifluoroacetamide (pK_a = 6.3) (Bordwell, 1977) acidities. For Asp 375, a pK_a near 5 is expected based on pK_a values for typical carboxylic acids, while a value near 6 has been inferred from the activity vs pH profile of citrate synthase (Koseki & Srere, 1961). All of these pK_a values are simple estimates, which do not take into account potential changes in pK_a values upon binding of inhibitor to citrate synthase.

However, two conclusions can be drawn. First, the pK_a of Asp 375 is above that of the carboxylate inhibitors, and well below that of the carboxamides. Second, fluorine substitition moves the carboxylate pK_a further from that of Asp 375 and moves the amide pK_a closer to that of Asp 375.

Matching of pK_a values is believed to be an important criteria for low-barrier hydrogen bond formation (Tobin et al., 1995). It is thus perhaps surprising that no correlation is observed between pK_a matching and hydrogen bond length in the series of inhibitors in this study, though all of these hydrogen bonds are longer than that observed in the symmetrical and very strong hydrogen bond formed between water and hydroxide ion (Abu-Dari et al., 1979). However, some correlation can be made between pK_a matching and binding affinity. It is established that the strengths of hydrogen bonds increase with increasing basicity of the hydrogen bond acceptor and/or increasing acidity of the hydrogen bond donor (Stahl & Jencks, 1986). The strongest hydrogen bonds should thus be formed when the pK_a of the hydrogen bond donor matches the pK_a of the conjugate acid of the hydrogen bond acceptor. CMX has a pK_a closest to that of Asp 375 and binds tightest, even though an additional penalty must be paid to protonate a carboxylate at pH 8, where the inhibitor assays were done. The decreased binding of FCMX relative to CMX corresponds to just over 2 kcal/mol. About 1 kcal/mol is attributed to desolvation of fluorine, which does not participate in any hydrogen bonds when bound. This estimate is based on the 6-fold difference in aqueous/organic partition coefficients between acetic and fluoroacetic acids (Korenman & Sel'manshchuk, 1982). The additional kilocalorie or more of binding is attributed to decreased strengths of hydrogen bonds formed by the less basic α-fluorocarboxylate. In the amide series, the bindings of AMX and FAMX are almost identical. This is consistent with a 1 kcal/mol increase in strength of the hydrogen bond to Asp 375, counteracting the presumed 1 kcal/mol cost of desolvating fluorine.

We have shown previously that the K_i for acetonyldethia-CoA, differing from acetyl-CoA only by replacement of sulfur by a methylene group, does not differ measurably from the K_m for acetyl-CoA (Martin et al., 1994). The data of Table 1 would thus appear to not be influenced by the sulfur to methylene substitution. However, the K_i values reported in Table 1 were measured at pH 8.0, at which binding of CMX and FCMX requires protonation of Asp 375.

The binding might be expected to increase by 2−3 orders of magnitude at low pH, based on the predicted pK_a for Asp 375 of 5−6. However, it has been shown previously with CMX (Usher et al., 1994) and with the related inhibitor carboxymethyl-CoA (Kurz et al., 1992) that the K_i decreases

only about 10-fold with a 2 pH unit decrease in pH, rather than the expected 100-fold. This is probably due to simultaneous titration of multiple functional groups. The enhanced affinity of CMX to protonated citrate synthase relative to the affinity of acetyl-CoA indicated by the data of Table 1 is probably low by 1−3 orders of magnitude, depending on the exact pK_a of Asp 375 and the use of measured vs extrapolated K_i values at low pH. However, considerations regarding protonation of Asp 375 do not affect comparisons of CMX vs FCMX and AMX vs FAMX discussed above. Amide analog binding does not change measurably upon lowering the pH from 8.0 to 7.0, supporting the assumption of amide binding with Asp 375 deprotonated.

Undoubtedly, changes in the pK_a of inhibitor also affect the strengths of hydrogen bonds with His 274 and the active site water molecule. The good correlation between binding affinities and pK_a matching with Asp 375 suggests that effects of pK_a matching on hydrogen bond strength may be felt most strongly in this unusually short hydrogen bond. However, the effects are small, about 1 kcal/mol for pK_a changes of 2−3 units, and the unusually short distance remains. These observations indicate that pK_a-matching is not critical to formation of the unusually short hydrogen bond. Nevertheless, it must fit some special set of criteria, since buried hydrogen bonds in proteins are seldom so short, and where exceptions such as these do arise, they are most often seen in active sites, with tight binding inhibitors, such as the ones discussed here, or phosphonate inhibitors of zinc proteases. The short distance could be enforced by other binding interactions between enzyme and inhibitor. However, carboxymethyl-CoA (CMC), which has an extra atom between the carboxylate and CoA moieties relative to CMX, forms a similarly short hydrogen bond with Asp 375 (Usher et al., 1994). This suggests that this close contact is induced by binding interactions of the carboxylate itself, and not by more distant interactions, which would be expected to have different effects on binding of CMX relative to its homolog CMC. Furthermore, all four inhibitors bind with substantially greater affinity than acetyl-CoA, which forms all of the same interactions as the inhibitors except for forming no hydrogen bond to Asp 375. This indicates that the hydrogen bonds to Asp 375 are all strong positive interactions, not negative interactions enforced by other binding interactions.

The strongest hydrogens bonds are expected to be formed when pK_a's are matched precisely while all of the inhibitors studied appear to differ from the pK_a of Asp 375 by 2 pK_a units or more. It is thus likely that none of the short hydrogen bonds formed in the citrate synthase−inhibitor complexes are optimal and that even stronger short hydrogen bonds may result from more precise pK_a matching. The estimated differences in hydrogen bond strengths with changes in the pK_a of hydrogen bonding groups are quite significant, though much smaller than differences observed in the gas phase. Furthermore, the changes in pK_a values and hydrogen bond strength do not seem to correlate to any detectable changes in hydrogen bond length. These results suggest caution in any attempts to correlate hydrogen bond strength with hydrogen bond length in enzyme−inhibitor complexes.

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