Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1981a) *FEBS Lett. 123*, 145–148.
Sottrup-Jensen, L., Lønblad, P. B., Stepanik, T. M., Petersen, T. E., Magnusson, S., & Jörnvall, H. (1981b) *FEBS Lett. 127*, 167–173.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1981c) *FEBS Lett. 128*, 123–126.
Steinbuch, M., Pejaudier, L., Quentin, M., & Martin, V. (1968) *Biochim. Biophys. Acta 154*, 228–231.
Straight, D. L., & McKee, P. A. (1982) *Biochemistry 21*, 4550–4556.
Straight, D. L., & McKee, P. A. (1984) *J. Biol. Chem. 259*, 1272–1278.
Strickland, D. K., & Bhattacharya, P. (1984) *Biochemistry 23*, 3115–3124.
Swenson, R. P., & Howard, J. B. (1979a) *J. Biol. Chem. 254*, 4452–4456.
Swenson, R. P., & Howard, J. B. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Swenson, R. P., & Howard, J. B. (1980) *J. Biol. Chem. 255*, 8087–8091.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655–709.
Van Leuven, F., Cassiman, J.-J., & Van der Berghe, H. (1981) *J. Biol. Chem. 256*, 9023–9027.
Walsh, K. A., & Neurath, H. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 884–889.
Wu, K., Wang, D., & Feinman, R. D. (1981) *J. Biol. Chem. 256*, 10409–10414.
Yamamoto, M., & Ikenkar, T. (1967) *J. Biochem. (Tokyo) 62*, 141–149.
Yung, B. Y., & Trowbridge, C. G. (1975) *Biochem. Biophys. Res. Commun. 65*, 927–930.

Conformations of Bound Nucleoside Triphosphate Effectors in Aspartate Transcarbamylase. Evidence for the London–Schmidt Model by Transferred Nuclear Overhauser Effects†

Anirban Banerjee,‡ H. Richard Levy,*,§ George C. Levy,‡ and William W.-C. Chan‖

NIH Research Resource for Multi-Nuclei NMR and Data Processing, Department of Chemistry, and Biological Research Laboratories, Department of Biology, Syracuse University, Syracuse, New York 13210, and Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Received August 17, 1984

ABSTRACT: Transferred nuclear Overhauser effects were used to determine the conformations of ATP, CTP, and ITP bound to the regulatory site of aspartate transcarbamylase. The results are in accord with the predictions of the London–Schmidt model [London, R. E., & Schmidt, P. G. (1972) *Biochemistry 11*, 3136] and show that ATP and CTP bind in the anti conformation while ITP binds in the syn conformation.

Aspartate transcarbamylase (ATCase)[1] (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) catalyzes the formation of carbamyl-L-aspartate, the first compound unique to the biosynthetic pathway for pyrimidine nucleotides. This enzyme has also served as a test of many of our current ideas of regulation, allosterism, and cooperativity.

The structure of the enzyme is well understood from the application of an array of techniques including X-ray diffraction, electron microscopy, amino acid sequence analysis, spectroscopic methods, and biochemical modifications (Jacobsen & Stark, 1973; Monaco et al., 1978; Kantrowitz et al., 1980a,b; Honzatko et al., 1980). The enzyme has a molecular weight of 300 000. It consists of six catalytic subunits (c) of molecular weight 33 000, arranged into two trimers (c_3), and six regulatory subunits (r) of molecular weight 17 000, arranged into three dimers (r_2). Zinc ions are present and play a structural role.

In *Escherichia coli*, ATCase is subject to activation by ATP and feedback inhibition by CTP, the end product of the pathway. These regulatory effects help to maintain the vital balance between purine and pyrimidine nucleotide pools necessary for DNA synthesis. Both these effectors bind to the same site on the regulatory dimer with ATP prompting the enzyme to assume an active R (relaxed) state, but CTP an inactive T (taut) state. Nucleoside triphosphate binding to

† This work was supported by grants from the Division of Research Resources, NIH (RR 01317 to G.C.L.), the National Science Foundation (PCM 8309379 to H.R.L.), and the Medical Research Council of Canada (W.W.-C.C.).

‡ Department of Chemistry, Syracuse University.
§ Department of Biology, Syracuse University.
‖ McMaster University.

[1] Abbreviations: ATCase, aspartate transcarbamylase; c–r unit, one peptide chain of a catalytic (c) subunit plus one peptide chain of a regulatory (r) subunit; E, enzyme; L, ligand (effector); ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; ITP, inosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; PALA, *N*-(phosphonoacetyl)-L-aspartate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TRNOE, transferred nuclear Overhauser effect; τ_c, reorientational correlation time; T, telsa; mG, milligauss; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay.

0006-2960/85/0424-1593$01.50/0

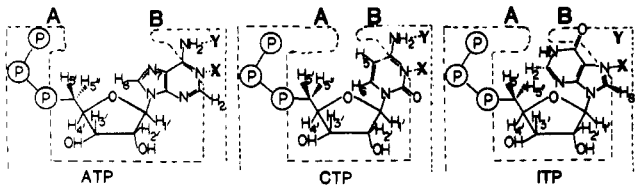

FIGURE 1: Expanded (more active enzyme) and contracted (less active enzyme) regulatory effector binding sites, according to the London–Schmidt model.

the regulatory subunits promotes conformational changes that are relayed to the catalytic subunits which themselves are insensitive to allosteric effectors. The actual binding of the inhibitor CTP to the six possible effector sites is exceedingly complex with heterotopic cooperativity also manifest (Jacobsen & Stark, 1973; Kantrowitz et al., 1980a,b).

London & Schmidt (1972) proposed a model of nucleoside triphosphate binding to ATCase from studies with various nucleotides and related compounds that affect the velocity of the enzyme-catalyzed reaction (Figure 1). Two subsites for nucleotide binding were invoked with subsite A binding the ribose triphosphate moiety and subsite B interacting with polar groups on the base. A strongly electrophilic group, X, at subsite B, is considered to react with a basic ring nitrogen of the nucleotide. With their similarity in structure, ATP and CTP can bind in an almost identical manner except that the smaller pyrimidine base of CTP is assumed to cause a contraction at the binding site, drawing A and B closer together, while the larger purine base of ATP presumably causes an expansion at this binding site. N3 of CTP is thought to interact with group X. For ATP, N1 (not N7) is the most basic ring nitrogen and is postulated to bind to X. Thus, both nucleoside triphosphates should bind in an anti conformation. Conversely, the weak inhibitors ITP and GTP should adopt a syn conformation while bound to this site in order to bring their most nucleophilic nitrogen atom, N7, close to X.

Lipscomb and co-workers (Honzatko et al., 1979; Honzatko & Lipscomb, 1982) have published crystallographic studies at 3-Å resolution that provide direct evidence of nucleoside triphosphate binding to the same regulatory site, with extended triphosphate groups. ATP, 5-BrCTP, and 8-BrGTP were shown to bind to the regulatory site of the enzyme in the anti conformation. They inferred that CTP and GTP also bind in the anti conformation like their bromo analogues. Although the anti conformation of bound ATP and the inferred anti conformation of bound CTP are consistent with the London–Schmidt model, the inferred anti conformation of bound GTP is not. Honzatko & Lipscomb (1982) suggested a modification of the London–Schmidt model to accommodate this apparent discrepancy.

NMR methodology has been successfully used in various studies on ATCase (Schmidt et al., 1969; Sykes et al., 1970; Moore & Browne, 1980) and has contributed significantly to our current understanding of this enzyme. Initially, TRNOE measurements (Bothner-By & Gassend, 1973; James & Cohn, 1974; James, 1976) were used to establish the proximity of bound ligands to particular residues on a protein. TRNOEs involving bound ligand resonances were first applied by Albrand et al. (1979) to a case where exchange between free and bound ligands was slow on the NMR time scale. TRNOE measurements have the unusual ability to study the conformation of flexible ligands (substrate and effector molecules) while in the *bound* state. The underlying theory of transferred NOEs (with ligand in fast exchange conditions included) has been described (Clore & Gronenborn, 1982, 1983). Unlike most NMR techniques, it is enhanced by the violation of the extreme narrowing condition due to slow molecular tumbling (long reorientational correlation times of the bound ligand). Our previous experience in analyzing conformations in exchanging systems (Levy et al., 1983) prompted us to examine the conformation of ATP and CTP bound to *E. coli* ATCase under realistic, dynamically exchanging solution conditions.

Within the extreme narrowing limit ($\tau_c < 10^{-10}$ s), proton–proton NOEs are positive with a maximum value of +0.5. In the spin diffusion limit ($\tau_c > 10^{-8}$ s), NOEs are negative, with a maximum value of −1. TRNOE uses chemical exchange to transfer information concerning cross-relaxation between two nuclei from the bound to the free state. Thus, negative NOEs on easily detectable free or observed ligand resonances are measured following irradiation of other ligand resonances (free, bound, or observed) in order to obtain conformational information in the bound state.

Under fast exchange conditions on the chemical shift scale, the individual position of a molecular resonance (I) in two different environments (I_f and I_b with f and b denoting the free and bound states, respectively) averages to a single peak (I_{obsd}). Relaxation due to dipolar coupling to S, a nearby nucleus on the same molecule (interproton distance $r < 4$ Å), generates the TRNOE whose normalized ($M_{f0} + M_{b0} = 1$) magnitude, N_I^{obsd}, at the averaged resonance, I_{obsd}, is

$$N_I^{obsd}(S) = M_{If,\infty} + M_{Ib,\infty} - 1$$

where M_{f0} and M_{b0} are the equilibrium magnetizations in the absence of any irradiation and $M_{If,\infty}$ and $M_{Ib,\infty}$ are the steady-state magnetizations of I following long irradiation such that M_S is saturated. A single negative TRNOE observation provides only qualitative structural information indicating that two ligand resonances are reasonably close together in the bound state.

Materials and Methods

Materials. Aspartate transcarbamylase was prepared according to the method of Gerhart & Holoubek (1967). ATP (P-L Biochemicals), CTP and ITP (Sigma Chemical Co.), GTP (Calbiochem), and D_2O (Stohler Isotope Chemicals) were obtained from the sources indicated. PALA was supplied by the National Cancer Institute, U.S. Public Health Service.

Methods. Enzyme (by extensive dialysis) and ligands (by repeated lyophilization) were exchanged into D_2O buffer (100% D_2O for the final exchange) to minimize the background HDO signal. The enzyme was first dialyzed vs. 40 mM potassium phosphate, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7 at 4 °C. In subsequent dialyses, 2-mercaptoethanol was omitted. Nucleoside triphosphates were added to give an approximately 15-fold ratio of free to enzyme bound, calculated from dissociation constants reported for ATP and CTP (Gray et al., 1973) and from approximate binding constants for ITP and GTP (London & Schmidt, 1972).

Transfer NOE experiments and other NMR studies were performed on a Bruker wide-bore WM-360 instrument with standard 5-mm NMR tubes. The observation of single, somewhat broadened (line widths between 8 and 25 Hz) ligand resonances in the presence of enzyme indicated there was fast averaging of chemical shift and line width between enzyme-bound and free states. The experimental approach (Clore & Gronenborn, 1982) was to first irradiate the methylene region (2.8 ppm) of the protein to acquire a control spectrum. Selected peaks were then irradiated; subtracting the control yielded difference spectra showing the extent of TRNOE on other spatially proximate resonances. The pulsing scheme applied was t_1–t_2–$\pi/2$–AT, where the delay t_1, inserted to eliminate spin diffusion through the protein, was empirically

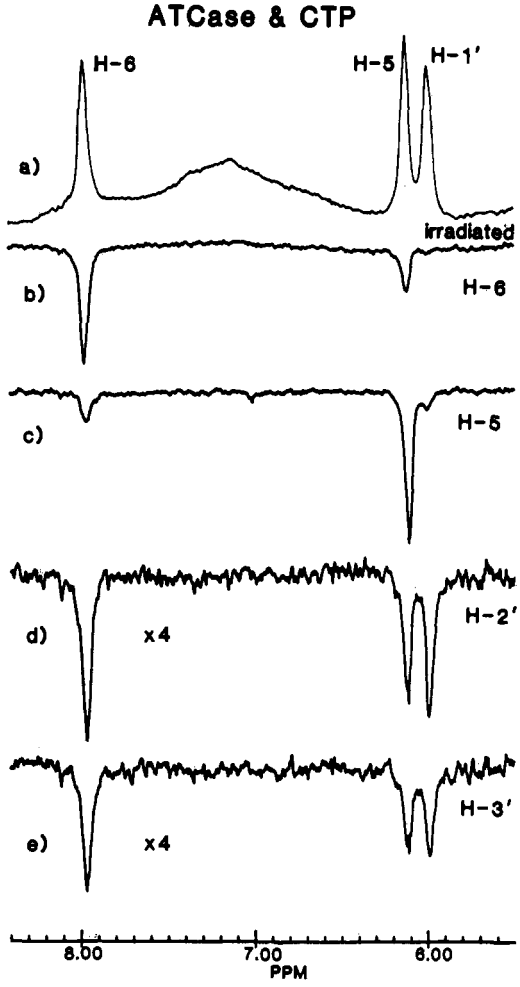

FIGURE 2: TRNOEs in CTP + ATCase evident from difference spectra. (a) Control spectrum with irradiation at 2.8 ppm on protein showing averaged ligand and broad envelope of amide and aromatic resonances. Spectra b–e were obtained by subtracting the control spectrum (a) from selectively irradiated spectra as indicated.

set between 0.7 and 1.8 s depending on the particular system. The single frequency irradiation time, t_2, was 0.44 s for these experiments. The saturating frequence was applied through the decoupler channel with typically 60 mG (25 Hz) of radio-frequency power. The acquisition time, AT, was 0.157 s in all cases. Resolution was not an important parameter, and extensive shimming or sample spinning was not required. Repetitive cycling of FIDs corresponding to various decoupling frequencies (typically every 200–500 scans) was performed to average long-term changes in spectrometer response. All shifts were referenced from the HDO signal taken as 4.8 ppm. Effector resonance assignments were confirmed by decoupling experiments on the pure compounds in buffer. These agreed with literature values where the latter were available. Representative TRNOE difference spectra for CTP with ATCase are shown in Figure 2.

TRNOEs were calculated by taking the ratio $[I(t) - I(0)]/I(0)$, the fractional change in intensity of the observed peak measured during selective irradiation of an averaged ligand resonance $I(t)$, from the intensity obtained during control irradiation on the protein envelope alone, $I(0)$. The ratio of two TRNOEs of spin I from irradiation of spins S and T is given by Clore & Gronenborn (1982) as

$$N_I(S)/N_I(T) \approx (r_{IT}/r_{IS})^6$$

This result provides estimates of relative internuclear distances. The structural information obtained from observing a single negative TRNOE will, of course, be of a qualitative nature, simply allowing one to ascertain that two ligand resonances are reasonably close together in the bound state.

Table I: TRNOE Measurements of CTP Bound to Aspartate Transcarbamylase with and without PALA

	normalized TRNOE on obsd resonances[a]					
	H_C6		H_C5		H_C1'	
irradiated resonance	E + CTP[b]	E + CTP + PALA[c]	E + CTP[b]	E + CTP + PALA[c]	E + CTP[b]	E + CTP + PALA[c]
H_C1'	*d*	*d*	*d*	*d*		
H_C2'	0.28	0.33	0.16	0.15	0.16	0.16
H_C3'	0.15	0.16	0.10	0.09	0.09	0.07
H_C4', 5', 5''[f]	0.28	0.23	0.18	0.14	0.20	0.16
H_C5	0.16	0.20			*e*	*e*
H_C6			0.22	0.28	*e*	*e*

[a] All TRNOEs are negative. [b] Enzyme concentration was 0.25 mM in c–r units, and CTP concentration was 7.8 mM. Selective saturation was applied with about 60 mG of radio-frequency power for 0.44 s. Relaxation delay t_1 was 1.6 s. [c] Conditions same as in footnote *b* except CTP concentration was 10.0 mM and 0.25 mM PALA was added. [d] Not measurable. Calculated to be smaller than 0.02. [e] Not measurable. Calculated to be smaller than 0.03. [f] H4' and H5', 5'' were not resolved.

Results

The data shown in the tables are averages of TRNOE values obtained from independent experiments performed on different days. The data from two separately prepared enzyme samples with CTP (agreeing within 3%) were averaged. After PALA was pipetted directly into these two tubes, two measurements were made on each sample. The four data sets thus obtained (agreement better than 4%) were averaged. An identical procedure was followed for ATP. For ITP and GTP, the data were obtained by averaging values from two independent experiments on a single sample of enzyme and nucleoside triphosphate (TRNOEs agreeing withing 3%).

Subtracting the control spectrum (obtained by irradiating the protein envelope) from other selectively irradiated spectra eliminates the indirect cross-relaxation of ligand resonances by protein spins. When control irradiation is applied to the methyl region of ATCase, small, negative TRNOEs (magnitudes less than 0.06) are observed for ATP resonances, especially on H2, but none of any significance is observed when the control irradiation is set as usual at the methylene region. As there are no ligand resonances near H2 of ATP to cause intraligand dipolar relaxation, it is possible that methyl groups are present in the vicinity of H2 in the regulatory site causing protein–ligand cross-relaxation. CTP gave the same values of TRNOE irrespective of the control irradiation position in the protein envelope.

Total normalized TRNOE on CTP H6 and ATP H8 is close to −1 (the predicted value for the very large ATCase molecule) but less for the other resonances measured: CTP H5, ribose H1', and, of course, ATP H2. The probable cause is again cross-relaxation with nonligand protons on protein and bound water.

Discussion

Table I lists the TRNOEs for the base-line-resolved CTP resonances H6, H5, and H1', generated by the irradiation of various sugar protons. The magnitudes of the TRNOE, which are proportional to r^{-6}, where r is the interproton distance, indicate that the pyrimidine protons H6 and H5 are situated over the ribose ring close to H2', H5', and H3'. As expected, the magnitudes of TRNOEs on cytidine H6 are larger than

Table II: TRNOE Measurements of ATP Bound to Aspartate Transcarbamylase with and without PALA

	normalized TRNOE on obsd resonances[a]					
	H_A8		H_A2		H_A1'	
irradiated resonance	E + ATP[b]	E + ATP + PALA[c]	E + ATP[b]	E + ATP + PALA[c]	E + ATP[b]	E + ATP + PALA[c]
H_A1'	0.05	0.06	*d*	*e*		
H_A2'	0.30	0.35	*d*	*e*	0.18	0.16
H_A3'	0.19	0.21	*d*	*e*	0.12	0.11
H_A4'	0.12	0.14	*d*	*e*	0.14	0.13
H_A5', 5''[f]	0.12	0.16	*d*	*e*	0.09	0.08
H_A8			*d*	*e*	0.07	0.03

[a] All TRNOEs are negative. [b] Enzyme concentration was 0.25 mM in c–r units, and ATP concentration was 4.3 mM. Selective saturation was applied with about 60 mG of radio-frequency power for 0.44 s. Relaxation delay t_1 was 1.6 s. [c] Conditions same as in footnote *b* except ATP concentration was 4.68 mM and 0.25 mM PALA was added. [d] Not measurable. Calculated to be smaller than 0.02. [e] Not measurable. Calculated to be smaller than 0.03. [f] H5′, 5″ were not resolved.

Table III: TRNOE Measurements of ITP Bound to Aspartate Transcarbamylase

	normalized TRNOE on obsd resonances[a,b]		
irradiated resonance	H_I8	H_I2	H_I1'
H_I1'	0.08	*c*	
H_I2'	0.26	0.04	0.18
H_I3'	0.12	0.05	0.12
H_I4'	0.07	0.05	0.15
H_I5', 5''[d]	0.12	0.08	0.11
H_I8		*c*	0.05

[a] All TRNOEs are negative. [b] Enzyme concentration was 0.5 mM in c–r units, and ITP concentration was 2.1 mM. Selective saturation on was decoupler channel used about 60 mG of radio-frequency power for 0.44 s. Relaxation delay t_1 was 1.6 s. [c] Not measurable. Calculated to be smaller than 0.02. [d] H5′, 5″ were not resolved.

corresponding H5 values (–0.28 and –0.33 vs. –0.16 and –0.15 from irradiating H2′ for instance), indicating that H6 is closer to those ribose protons than is H5 and furthest from H1′, from which there is no measurable TRNOE. Also, the TRNOE on H6 when H5 is irradiated (–0.16 and –0.20) is less than the TRNOE generated on H5 when H6 is irradiated (–0.22 and –0.28), as H5 is relatively isolated from sugar protons, while H6 has H2′, H3′, and H5′,5″ along with the base H5 proton as close cross-relaxation partners. Since the observed Overhauser enhancement arises from the ratio of selective cross-relaxation to total relaxation, the relaxation of H6 due to H5 alone is decreased by the simultaneous presence of competetive cross-relaxation to the other nearby protons.

For ATP, TRNOE values in Table II show that the adenine H8 proton (like CTP H6 and H5) is quite close to the ribose H2′ (–0.30 and –0.35), H3′ (–0.19 and –0.21), and H5′,5″ (–0.12 and –0.16). Distances between H4′–H8 and H4′–H1′ protons are similar. Base proton H2, on the other hand, is too distant from sugar protons for TRNOEs to be measurable.

In Table III, the results for the weak inhibitor ITP (London & Schmidt, 1972) show that the purine H8 proton is close to ribose H2′ and H3′, but in contrast with ATP, there is a significant TRNOE observed on H2 from the various ribose protons, especially H5′,5″. Therefore, H8 must be clear of the ribose ring lying somewhere between H2′ and H1′ (Figure 3). Note that H8 and H1′ each show approximately equal TRNOEs when H3′ and H5′,5″ are irradiated, confirming this inference.

By building models from the ratios of internuclear distances, it can be found that the ATP purine and CTP pyrimidine bases are in an anti [specifically, an *ac* (anticlinal)] conformation

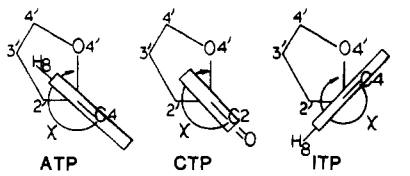

FIGURE 3: Schematic projections of effectors showing probable conformations about the N-glycosidic bond when bound to the regulatory site. Positions with the dihedral angle χ between 90° and 270° are considered anti. ATP is viewed down the N9–C1′ bond and is shown as *ac* (anticlinal); CTP is viewed down the N1–C1′ bond and is also shown in the *ac* conformation; ITP is viewed down the N9–C1′ bond and is shown in the *sc* (synclinal) conformation.

Table IV: TRNOE Measurements of GTP Bound to Aspartate Transcarbamylase

	normalized TRNOE on obsd resonances[a,b]	
irradiated resonance	H_G8	H_G1'
H_G1'	0.04	
H_G2'	0.16	0.11
H_G3'	0.09	0.07
H_G4'	0.06	0.10
H_G5', 5''[c]	0.07	0.08
H_G8		0.05

[a] All TRNOEs are negative. [b] Enzyme concentration was 0.5 mM in c–r units, and GTP concentration was 4.0 mM. Selective saturation on the decoupler channel used about 60 mG of radio-frequency power for 0.44 s. Relaxation delay t_1 was 1.6 s. [c] H5′, 5″ were not resolved.

Table V: TRNOE Ratios in Effectors Bound to Aspartate Transcarbamylase

irradiated resonance	H6/H1′, CTP	H8/H1′			H2/H1′	
		ATP	ITP	GTP	ATP	ITP
H2′	1.9	1.9	1.4	1.4	<0.1	0.2
H3′	2.0	1.8	1.0	1.3	<0.2	0.4
H4′	1.4	1.0	0.5	0.6	<0.1	0.3
H5′, 5″		1.7	1.1	1.0	<0.2	0.7

[a] H4′ and H5′, 5″ were not resolved.

with a glycosidic angle χ ~240° as shown schematically in Figure 3. The evidence for ITP suggests a syn [specifically, a *sc* (synclinal)] conformation, with glycosidic angle χ ~330°. TRNOEs observed when H1′ is irradiated are too small in all cases and thus were not used to calculate distance ratios for building models. The similarity in TRNOE values from ribose H2′ to H1′ and from H3′ to ATP H8 or CTP H6 (implying an approximate equality in distances), together with the ratios of H2′, H3′, and H4′ to H1′, suggests that ribose in the effectors ATP and CTP (and probably also ITP) is in the N (3′-endo, 2′-exo) conformation. Though the H5′,5″ protons are not resolved, the larger TRNOE from these to the nearest base protons rather than to H1′, together with other distance ratios, suggests a conformation about the C4–C5 bond that is more trans (trans-gauche, gauche-trans, or gauche-gauche), than cis (Clore & Gronenborn, 1983).

Table IV lists results with another weak purine inhibitor, GTP; however, guanine lacks the H2 proton, critical for comparative TRNOE studies. The data *suggest* proximity of H8 and ribose H2′ and H3′, although it is not clear whether H8 lies over the plane of the ribose ring near H2′, H3′, and H5′,5″ like H8 in ATP (anti, glycosidic angle χ ~240°) or whether it lies between H1′ and H2′ (syn, glycosidic angle χ ~330°). If the model conformation is assumed to be anti, the lowered TRNOEs from H5′,5″ to H8 require the H5′,5″ protons to be further away from H2′, H3′, and H8 with conformation about the C4′–C5′ bond of the guanosine ribose more cis than the usual trans. Moreover, the ribose confor-

mation itself is then likely to be between the N and S types rather than clearly N. This is similar to the conformation for 8-BrGTP found by Honzatko & Lipscomb (1982). If the model conformation is assumed to be syn, the conformation of GTP would be similar to that of ITP, consistent with their similar structures, binding characteristics, and weak inhibitory effects. Although the data for the inhibitor GTP are ambiguous with respect to whether the conformation is *ac* or *sc*, they do not contradict the London–Schmidt model, according to which GTP (like ITP) should bind in the syn conformation. The ratios shown in Table V of TRNOE to H8 and H1′, from ribose protons H2′, H3′, H4′, and H5′,5″, are very similar for ITP and GTP and differ significantly from those seen for ATP. However, the existence of more than one conformation (and an exchange between them) cannot be ruled out; averaging between interproton distances and consequent cross-relaxation would affect the measured TRNOEs.

On the basis of electron density maps obtained at 3-Å resolution, Honzatko et al. (1979) reported that the allosteric site of *E. coli* ATCase binds ATP in the anti conformation and 5-BrCTP in the syn conformation. They postulated that CTP, like 5-BrCTP, also binds in the syn conformation. Subsequently, Honzatko & Lipscomb (1982), applying improved phase estimates to the earlier data, corrected the earlier conformation assignment of bound 5-BrCTP from syn to anti. They were able to fit CTP to the electron density by assuming that the interactions of CTP and 5-BrCTP with enzyme were identical and thus concluded that CTP was also bound in the anti conformation (Honzatko & Lipscomb, 1982). Binding of ATP and CTP in the anti conformation is consistent with the model of London & Schmidt (1972) for nucleoside triphosphate binding to the allosteric site of ATCase. However, London and Schmidt also proposed that the weak inhibitors GTP and ITP bind to the same site in the syn conformation due to the specific interaction between an electrophilic moiety, X, on the protein and N7, the most basic nitrogen of these nucleoside triphosphates. On the basis of difference map data from 8-BrGTP, Honzatko & Lipscomb (1982) suggested that GTP binds to the allosteric site of ATCase in the anti conformation. Their model of 8-BrGTP binding, however, invokes a mode of interaction between N7 on the purine ring and regions of the enzyme that are distinct from those that bind ATP or CTP (Honzatko & Lipscomb, 1982). This postulated binding mode is different from that suggested by London & Schmidt (1972).

Our results based on nonbrominated nucleoside triphosphates avoid the need to make any assumptions about identical modes of binding of brominated and nonbrominated nucleotides. The data show that ATP and CTP are bound to ATCase in the anti conformation and that ITP is bound in the syn conformation. We are unable to assign the conformation of bound GTP unequivocally because it lacks H2, but the data are consistent with a syn conformation according to the London–Schmidt model, as well as the new anti conformation proposed by Honzatko & Lipscomb (1982) including the rotated C4′–C5′ bond conformation.

PALA, a transition-state analogue that binds tightly to the substrate binding site (Collins & Stark, 1971), was added to block this binding site (Suter & Rosenbusch, 1977), and hence prevent weak nucleoside triphosphate binding. The presence of PALA does not seem to alter effector conformation significantly as can be seen by comparing the relevant columns in the tables. However, the binding of PALA at one of the six catalytic sites of ATCase causes a conformational change (similar to the one caused by the binding of both substrates) to the R, active state (Howlett et al., 1977). CTP inhibits the enzyme and promotes the inactive T state but also binds to the R state, though at a different rate (Howlett et al., 1977; Gray et al., 1973). The addition of PALA reveals the scalar coupling between CTP resonances H6 and H5. The peaks appear without discernible line sharpening as poorly resolved doublets with 6.1-Hz separation (at magnetic field strengths of 8.4 and 5.87 T and which can be decoupled to singlets). The reason why addition of PALA allows the coupling to be observed may involve changes in the chemical shift or changes in the residence times in environments between which the ligand is exchanging, or both. The sugar resonances are too broad (20–30 Hz) to show any coupling themselves.

In conclusion, our data support the predictions of the London–Schmidt model. PALA itself causes no dramatic changes of effector conformation, in keeping with the notion that regulatory site geometry is unaffected by catalytic site occupation (Chan, 1975). The extent of the TRNOE generated is dependent on exchange rates, molecular motion in the bound state (hence, cross-relaxation rates), and enzyme structure as well as the experimental parameters of irradiation power and duration, relaxation delays, etc. Nevertheless, TRNOE spectroscopy can serve as a valuable auxiliary alternative to x-ray methods and in suitable cases can supply information otherwise unavailable about dynamically exchanging systems.

ACKNOWLEDGMENTS

We acknowledge helpful discussions with Dr. Andrzej Ejchart.

REFERENCES

Albrand, P. L., Birdsall, B., Feeney, J., Roberts, G. C. K., & Burgen, A. S. V. (1979) *Int. J. Biol. Macromol. 1*, 37.

Bothner-by, A. A., & Gassend, R. (1973) *Ann. N.Y. Acad. Sci. 222*, 668.

Chan, W. W.-C. (1975) *J. Biol. Chem. 250*, 668.

Clore, G. M., & Gronenborn, A. M. (1982) *J. Magn. Reson. 48*, 402.

Clore, G. M., & Gronenborn, A. M. (1983) *J. Magn. Reson. 53*, 423.

Collins, K. D., & Stark, G. R. (1971) *J. Biol. Chem. 246*, 6599.

Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886.

Gray, C. W., Chamberlain, M. J., & Gray, D. M. (1973) *J. Biol. Chem. 248*, 6071.

Honzatko, R. B., & Lipscomb, W. N. (1982) *J. Mol. Biol. 160*, 265.

Honzatko, R. B., Monaco, H. L., & Lipscomb, W. N. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5105.

Howlett, G. J., Blackburn, M. N., Compton, J. G., & Schachman, H. K. (1977) *Biochemistry 16*, 5091.

Jacobsen, G. R., & Stark, G. R. (1973) *Enzymes, 3rd Ed. 9*, 225.

James, T. L. (1976) *Biochemistry 15*, 4724.

James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 2599.

Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980a) *Trends Biochem. Sci. (Pers. Ed.) 5*, 124.

Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980b) *Trends Biochem. Sci. (Pers. Ed.) 5*, 150.

Levy, H. R., Ejchart, A., & Levy, G. C. (1983) *Biochemistry 22*, 2792.

London, R. E., & Schmidt, P. G. (1972) *Biochemistry 11*, 3136.

Monaco, H. L., Crawford, J. L., & Lipscomb, W. N. (1978)

Proc. Natl. Acad. Sci. U.S.A. 75, 5276.
Moore, A. C., & Browne, D. T. (1980) *Biochemistry 19*, 5768.
Schmidt, P. G., Stark, G. R., & Baldeschwieler, J. D. (1969) *J. Biol. Chem. 244*, 1860.
Suter, P., & Rosenbusch, J. P. (1977) *J. Biol. Chem. 252*, 8136.
Sykes, B. D., Schmidt, P. G., & Stark, G. R. (1970) *J. Biol. Chem. 245*, 1180.

Aspartate β-Decarboxylase from *Alcaligenes faecalis*: Carbon-13 Kinetic Isotope Effect and Deuterium Exchange Experiments†

Robert M. Rosenberg‡ and Marion H. O'Leary*
Departments of Chemistry and Biochemistry, University of Wisconsin, Madison, Wisconsin 53706
Received July 24, 1984; Revised Manuscript Received October 30, 1984

ABSTRACT: We have measured the ^{13}C kinetic isotope effect at pH 4.0, 5.0, 6.0, and 6.5 and in D_2O at pD 5.0 and the rate of D–H exchange of the α and β protons of aspartic acid in D_2O at pD 5.0 for the reaction catalyzed by the enzyme aspartate β-decarboxylase from *Alcaligenes faecalis*. The ^{13}C kinetic isotope effect, with a value of 1.0099 ± 0.0002 at pH 5.0, is less than the intrinsic isotope effect for the decarboxylation step, indicating that the decarboxylation step is not entirely rate limiting. We have been able to estimate probable values of the relative free energies of the transition states of the enzymatic reaction up to and including the decarboxylation step from the ^{13}C kinetic isotope effect and the rate of D–H exchange of α-H. The pH dependence of the kinetic isotope effect reflects the pK_a of the pyridine nitrogen of the coenzyme pyridoxal 5′-phosphate but not that of the imine nitrogen. A mechanism is proposed for the exchange of aspartate β-H that is consistent with the stereochemistry suggested earlier.

Aspartate β-decarboxylase catalyzes the decarboxylation of L-aspartic acid to alanine, as in eq 1. Both a keto acid,

$$^{-}OOC\text{–}CH_2CH(NH_3^+)\text{–}COO^- + H^+ \rightarrow CO_2 + CH_3CH(NH_3^+)\text{–}COO^- \quad (1)$$

in this work α-ketoglutaric acid, and the coenzyme pyridoxal 5′-phosphate must be added to the enzyme before the addition of substrate to prevent abortive transamination (Tate & Meister, 1969). The accepted mechanism for the reaction (up to the decarboxylation step), based principally on the work of Meister and his collaborators, is shown in Scheme I (Tate & Meister, 1969; Chang et al., 1982). This enzyme is different from other pyridxal phosphate dependent decarboxylases whose kinetic isotope effects have been studied (O'Leary et al., 1970, 1981; O'Leary & Piazza, 1981) in that there is a hydrogen-transfer step prior to the decarboxylation step.

One goal of the enzymologist is to deduce the complete free energy profile for the steps of an enzyme mechanism (Knowles & Albery, 1977; Albery & Knowles, 1976a; Ray, 1983). Such a determination would permit identification of the rate-limiting, or "most sensitive", step in the sequence (Ray, 1983) and estimation of the degree to which the enzyme has evolved to an optimum catalytic efficiency (Albery & Knowles, 1976b). Isotope effects are one particularly useful way to get this information (Knowles & Albery, 1977). Particularly when multiple isotope effects are available for a reaction, it can be possible to obtain a relatively complete free energy profile. We have been able to determine patterns for the relative free energy of each of the transition states for the decarboxylation of aspartic acid up to the irreversible loss of CO_2 through a combination of measurements of the rate of D–H exchange of the α-H of aspartic acid and measurements of the ^{13}C kinetic isotope effect on V/K.

Materials and Methods

Enzymes. L-Aspartic acid β-decarboxylase (specific activity 70 IU/mg) was prepared from *Alcaligenes faecalis*, strain N (AT CC 25094), by Aghdas Laghai (Chang et al., 1981) by the method of Tate et al. (1970). Glutamic-oxaloacetic transaminase and malic dehydrogenase were obtained from Sigma Chemical Co.

Chemicals. All the L-aspartic acid used was from a single bottle (to avoid variations in isotope composition) obtained from Aldrich Chemical Co. Pyridoxal 5′-phosphate, α-ketoglutaric acid, bovine serum albumin, reduced nicotinamide adenine dinucleotide, and hydroxypropylcellulose were obtained from Sigma Chemical Co. The D_2O used was 99.8 atom % D. Other chemicals were of reagent grade.

Enzyme Assay. Aspartate β-decarboxylase activity was determined respirometrically at 37.0 °C (Umbreit et al., 1972) with a Gilson constant-pressure respirometer. The method of Novogrodsky & Meister (1964) was used, with 2.5 mL of the following solution in the flask: 0.10 M acetate buffer, pH 5.0, 2 mM α-ketoglutaric acid, 0.86 mM pyridoxal 5′-phosphate,[1] and enzyme and with 0.5 mL of 4×10^{-2} M aspartic acid, titrated to pH 5.0, in the side arm. Because acetate ion was found to be an inhibitor of the enzyme (Novogrodsky & Meister, 1964; R. M. Rosenberg and M. H. O'Leary, un-

†This research was supported by Grant PCM 8216597 to M.H.O. from the National Science Foundation and by Lawrence University.
*Address correspondence to this author at the Department of Chemistry.
‡On sabbatical leave from Lawrence University, Appleton, WI 54912.

[1] The α-ketoglutaric acid and pyridoxal 5′-phosphate were added to prevent the abortive transamination and loss of coenzyme as pyridoxamine 5′-phosphate (Tate & Meister, 1969).

0006-2960/85/0424-1598$01.50/0