EVIDENCE FOR METHIONINE SULFOXIMINE AS A TRANSITION-STATE ANALOG FOR GLUTAMINE SYNTHETASE FROM NMR AND EPR DATA*

J. J. Villafranca,** D. E. Ash† and F. C. Wedler

Departments of Chemistry
The Pennsylvania State University
University Park, Pennsylvania 16802
and Renssalaer Polytechnic Institute
Troy, New York 12181

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SUMMARY: Manganese(II)bound at the "tight" metal ion site of unadenylylated glutamine synthetase (E. coli W) has two rapidly exchanging first coordination sphere water molecules. The solvation number was evaluated from a study of the frequency dependence of $1/pT_{1p}$, the paramagnetic contribution to the longitudinal relaxation rate of solvent protons. The number of rapidly exchanging water molecules is reduced to one in the presence of saturating L-glutamate and to ~0.2 when L-methionine SR-sulfoximine (MSOX) is present. MSOX is a linear competitive inhibitor (K_I=3μM) of glutamine synthetase when L-Glu is the substrate. The dissociation constant of MSOX measured by following the 18 fold decrease in $1/pT_{1p}$ (at 48 MHz) is 30μM and is lowered to ~9μM in the presence of ADP. The high affinity of MSOX for the enzyme suggests that this compound mimicks the "transition-state" for the glutamine synthetase reaction. Further evidence for this postulate is found from the dramatic sharpening of the epr spectrum of enzyme-bound Mn(II) in the presence of MSOX and MSOX plus ADP. The intense change in the epr spectrum arises from reduced solvent accessibility to bound Mn(II) and conformational changes produced by binding MSOX and ADP. The suggestion is made from these data that L-Glu and MSOX bind near or directly to the Mn(II) at the "tight" metal ion site in glutamine synthetase isolated from E. coli W.

Glutamine synthetase (GS) catalyzes the formation of glutamine from glutamate, NH_4^+ and ATP. The enzyme isolated from E. coli has twelve identical subunits with two Mn(II) binding sites of unequal affinity per subunit (Ginsburg, 1972; Hunt et al., 1975). The high affinity "tight" metal ion site (per subunit)

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** To whom to address inquiry.

† Present address, Chemistry Department, University of Illinois, Urbana, Illinois 61801.

is implicated in changing the protein conformation from relaxed to taut forms (Shapiro and Ginsburg, 1968), while the second "weak" metal ion site is implicated in catalysis since this site is the Mn-ATP site (Hunt et al., 1975). The "tight" metal ion site could also be involved in catalysis and/or binding by closely interacting with bound glutamate (Villafranca and Wedler, 1974). It is the purpose of this paper to present nuclear magnetic resonance (nmr) and electron paramagnetic resonance (epr) evidence which suggests that the substrate glutamate binds to GS near the "tight" metal ion site. Additional evidence is presented that L-methionine SR-sulfoximine is a transition-state analog for the GS reaction and intimately interacts with enzyme-bound Mn(II). Meister's group unequivocally demonstrated that inhibition of brain glutamine synthetase by methionine sulfoximine involved tight irreversible association with the enzyme and formation of methionine sulfoximine phosphate in the presence of ATP and metal ions (Ronzio and Meister, 1968; Ronzio et al., 1969; Rowe et al., 1969).

Materials and Methods

Glutamine synthetase was isolated from E. coli W in a state of low adenylylation ($E_{1.7}$) as described previously (Woolfolk et al., 1966). Buffers, L-glutamate, L-methionine SR-sulfoximine and L-methionine sulfone were products of Sigma. The enzyme activity was determined by the method of Hunt et al. (1975).

A Varian E-12 spectrometer was used to record spectra at 9 GHz. The spectrometer was equipped with a variable temperature unit and maintained the temperature within $\pm 1^{\circ}$. Aqueous samples of 25-40$\mu\ell$ were placed in quartz capillary tubing (1.0mm i.d.) and closed with a teflon plug. When aqueous demetalated enzyme samples were run, there were no observable signals from 1000 to 5000 gauss at the highest gain, power and modulation settings used for this work.

Details of the pulsed nmr spectrometer and data analysis are given in previous papers (Villafranca and Colman, 1974; Villafranca and Wedler, 1974).

Results and Discussion

Table I lists kinetic and nmr data taken with unadenylylated glutamine synthetase. The dissociation constant, K_3, of L-Glu from $E_{1.7}$-Mn(II) is 3.0mM. The technique used is outlined by Mildvan and Cohn (1970) and is essentially

Table I

Kinetic and Nuclear Magnetic Resonance Data

with Unadenylylated Glutamine Synthetase[a]

	GS-Mn(II)	GS-Mn(II)-L-Glu	GS-Mn(II)-MSOX
K, mM(kinetic)	--	3.2[b]	0.003[c]
K_3, mM(nmr)	--	3.0[d]	0.030[d] (0.009)[e]
$1/pT_{1p}$, sec^{-1} (6MHz)[f]	4.04×10^6	2.90×10^6	1.13×10^6
(24MHz)	6.63×10^6	4.35×10^6	0.76×10^6
(48MHz)	3.80×10^6	2.36×10^6	0.21×10^6
n[g]	1.9	1.1	0.2

[a] Glutamine Synthetase with an average state of adenylylation of 1.7 was used. In nmr experiments, 0.1 to 0.8mM enzyme in 10mM imidazole buffer, pH 7.2, containing 0.1 MKCl was used. The enzyme contained 0.9 mole of Mn(II) per mole of enzyme subunit. Under these conditions only the "tight" Mn(II) site was populated.

[b] K_m' value for L-glutamate from Denton and Ginsburg (1970).

[c] K_I value for L-methionine SR-sulfoximine (MSOX). MSOX was a linear competitive inhibitor with respect to L-gluatmate (F. C. Wedler, unpublished data).

[d] Determined from nmr titration experiments at 48MHz and 23° by following the decrease in the water proton relaxation rate ($1/T_1$). Typical titrations utilized 50$\mu\ell$ of 0.1mM enzyme with addition of 1$\mu\ell$ aliquots of L-Glu or MSOX. K_3 values were evaluated by the general method of Reed et al. (1970).

[e] Titration in the presence of 15mM ADP.

[f] All values determined at 25° (0.4mM GS). For GS-Mn(II)-L-Glu, 50mM L-Glu was present and for GS-Mn(II)-MSOX, 3mM MSOX.

[g] The method outlined by Villafranca and Wedler (1974) was used to compute n from the $1/pT_{1p}$ values.

following the decrease in the paramagnetic contribution to the longitudinal relaxation rate ($1/T_{1p}$) of water protons. This value is in close agreement with the Km' value of 3.2mM, determined by kinetic methods. The agreement is good considering the fact that the Km' value was determined in solutions which contained excess metal ions, ATP and NH_4^+. L-Methionine SR-sulfoximine (MSOX) is a linear-competitive inhibitor with respect to L-Glu with a K_I value of 3μM (Table I). This K_I value is about three orders of magnitude lower than the Km' value for L-Glu. This increased affinity for an inhibitor clearly falls within the definition of a transition-state analog (Wolfenden, 1972). The K_3 value of 30μM is about 100 fold weaker than the K_I value and decreases to 9μM in the presence of ADP. Ronzio and Meister (1968) suggested that MSOX can bind simultaneously at both the glutamate and ammonia binding sites and when phosphorylated (MXOX-P)(I) may resemble the postulated phosphorylated tetrahedral intermediate(II) in the glutamine synthetase reaction.

I (R is $-CH_2CH_2CH(NH_3^+)CO_2^-$) II

Table I also lists $1/pT_{1p}$ values at three frequencies for three complexes of GS ("tight" metal ion site occupied). The data for GS-Mn(II) and GS-Mn(II)-L-Glu show a frequency dependence with a contribution to the correlation time from the electron spin relaxation time of Mn(II) (Villafranca and Wedler, 1974). Analysis of the number of rapidly exchanging water molecules, n, (Table I) leads to the conclusion that L-Glu decreases n from ~2 to ~1. This suggests that L-Glu is near the "tight" Mn(II) site and either occludes one water molecule from rapid exchange or directly displaces one water molecule on GS-bound Mn(II). The presence of MSOX in solutions of GS-Mn(II) produces a dramatic drop in $1/pT_{1p}$ (greater than an order of magnitude at 48 MHz) and reduces n to ~0.2. These data are interpreted in terms of total obstruction of water exchange from bound Mn(II) by the presence of MSOX. The residual $1/pT_{1p}$ values could result from outer-sphere proton relaxation of water molecules near or on the surface of the protein.

The reduction of n from ~2 to ~0 by the binding of MSOX suggests that the epr spectrum of enzyme-bound Mn(II) should reflect these dramatic changes in water solvation. Fig. 1 shows epr spectra of GS-Mn(II) and complexes with L-Glu or MSOX. The top spectrum demonstrates that Mn(II) bound at the "tight" metal ion site of glutamine synthetase is in a relatively isotropic environment. The presence of L-Glu (Fig. 1, middle) produces a change in the spectrum with a new unresolved broad peak at a lower field position from the main six line pattern. This reflects a change in geometry about the Mn(II) which suggests that when L-Glu binds to GS-Mn(II) this substrate binds close to (if not directly to) the Mn(II). These data coincide with the nmr data in which n was reduced from ~2 to ~1.

When MSOX is added to GS-Mn(II), a dramatic change in the epr spectrum is

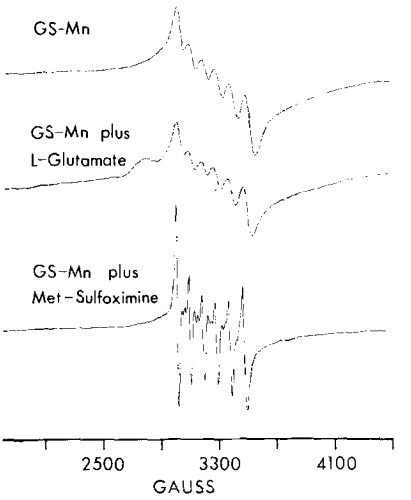

Figure 1. Electron Paramagnetic Resonance spectra of glutamine synthetase-bound Mn(II). The enzyme subunit concentration was 0.8mM with a Mn(II) concentration of 0.75mM. All spectra were taken at 1° under the following experimental conditions: 150mW power; 9.13 GHz; relative gain, 1 (top and middle), 0.5 (bottom); modulation amplitude, 25 gauss (top and middle). 5 gauss (bottom).

observed (Fig. 1, bottom). The line width of the epr transitions narrows from ~30 gauss peak to peak in GS-Mn(II) to ~ 12 gauss in GS-Mn(II)-MSOX. The transitions narrow to ~9 gauss peak to peak in the presence of ADP. This is precisely what is predicted if Mn(II) is inaccessible to rapid solvent collision, i.e., Mn(II) is in a "crystalline,""solid-state" environment (see Bloembergen and Morgan, 1961, and Hudson and Luckhurst, 1969, for discussion of Mn(II) electronic relaxation rates and Reed et al., 1971, Reed and Cohn, 1970, and Reed and Ray, 1971, for detailed explanations of Mn(II) epr spectra for macromolecules). The bound Mn(II) is in a cubic environment when MSOX is present and the less intense transitions found between the six major -1/2 to 1/2 transitions are most likely forbidden transitions. Similar spectra are observed for Mn(II) doped in cubic MgO crystals (Wolga and Tseng, 1964).

If MSOX is added to a solution of GS-Mn(II)-L-Glu, the epr spectrum is identical to the one shown at the bottom of Fig. 1. Addition of up to 100mM L-Glu could not revert the epr spectrum to the one obtained with L-Glu alone. This is consistent with the difference in magnitude in binding constants and with findings of Ronzio and Meister (1968). Addition of L-methionine sulfone to GS-Mn(II) did not produce dramatic changes.

The intense change in the epr spectrum upon addition of MSOX to GS-Mn(II) coupled with the decrease in n from ~2 to ~0 suggests that MSOX binds very close to the "tight" Mn(II) site. By bridging the ammonia and glutamate binding sites, MSOX may produce a conformational change in the protein resulting in the tight binding of the inhibitor (Table I). The tightening is enhanced by ADP and additional conformational changes seen in the epr spectra. This tightened binding is expected for compounds which resemble the transition state (Wolfenden, 1972) and provides the first evidence that the "tight" Mn(II) site may be involved in glutamate binding and/or catalysis.

The role of Mn(II) could be to provide an electrophilic site to polarize the carbonyl oxygen of γ-glutamyl phosphate for enhanced nucleophilic attack by ammonia (Villafranca and Wedler, 1974).

The tetrahedral sulfur atom of MSOX may be a good "mimick" of the transition-state of the glutamine synthetase reaction especially if the overall reaction is concerted (Wedler and Boyer, 1972) or has a discrete intermediate(s). For either case the structures I and II demonstrate how the "fit" in the active site may have substantial tetrahedral character during the reaction. Tate and Meister (1973) have pointed this out previously and used computer simulation to model the active site. Further experimentation is underway to study binding by methionine sulfoximine phosphate, substrates and other inhibitors.

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