BioMetals **13:** 195–201, 2000.

Lanthanide ions inhibit the activity of dihydrofolate reductase from chicken liver

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Received 15 March 2000; accepted 2 May 2000

Key words: DHFR, enzyme activation, enzyme inhibition, fluorescence probe, lanthanide elements

Abstract

The influences of mono-, bi- and trivalent metal ions (as chloride salts) on the activity of dihydrofolate reductase (DHFR) from chicken liver have been studied to elucidate the mechanism of ion-activation of this enzyme. The results show that monovalent ions (Na^+ and K^+) activate DHFR at low concentration reaching a maximum activation of about 2.5 fold at 0.4–0.5 M and declining at higher concentrations. Ca^{2+} shows similar activation but at lower concentration, reaching a maximum at 0.1 M; activity declines with further increases in concentration. At very high concentration (> 0.4 M), Ca^{2+} is inhibitory. The trivalent lanthanide ions, however, show a dramatic inhibition of activity of DHFR even at very low concentration. The activity of DHFR declines to 50% of that of the control at 0.02 mM $EuCl_3$. Intrinsic fluorescence measurements show that the ion-dependent activation in the presence of mono- and bivalent metal ions is due to the conformational changes in the protein. Energy transfer phenomenon suggests that the specific interaction of Eu^{3+} with Trp24 located in a loop at the active site of DHFR is responsible for the strong inhibition. The possible mechanism for the ion-inhibition is proposed and discussed.

Introduction

Dihydrofolate reductase (DHFR, EC 1.5.1.3) catalyzes the NADPH (reduced nicotinamide adenine dinucleotide phosphate)-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) and plays an important role in nucleotide biosynthesis. Therefore the activation and inhibition of DHFR by folate analogues (Cayley *et al.* 1981; Baccanari *et al.* 1982; Blackly 1984; Stone & Morrision 1984) have been extensively studied. One of the most interesting properties of eukaryotic DHFR is that the protein can be activated by a diverse group of agents (Blackly 1984), such as inorganic salts (Reyes & Huennkens 1967; Blackly 1984), mercurials (Kaufman 1964), sulfhydryl modifiers (Kaufman 1964; Barbehenn & Kaufman 1982; Duffy *et al.* 1987) and chaotropes such as urea and guanidine hydrochlorides (Kaufman 1968; Duffy *et al.* 1987; Fan *et al.* 1995). Although the activation by urea and mercurials has been studied in detail, the mechanism of ion-activation is still not fully understood.

The activation of DHFR from L1210 cells by salts (Na^+, K^+, Ca^{2+} as chlorides) had been studied and the effects of these salts were attributed to the common anion (Reyes & Huennkens 1967), the role of the cation is not clear. The activation of the enzyme by these salts was believed to involve conformational changes in the protein. The mechanism, i.e., En$\rightarrow$Ea$\rightarrow$Ei, where En, Ea and Ei designate the native, activated and inactive forms of the enzyme, respectively, was proposed to be involved in the ion-dependent activation process (Duffy *et al.* 1987). The enzyme was proposed to interconvert between a series of activated forms whose structures have small yet significant differences. Direct evidence for this mechanism was not possible since these metal ions all have closed electronic shells and therefore lack useful spectroscopic characteristics.

In this paper, lanthanide ions were used as ion probes to study the mechanism of ion-activation of

DHFR from chicken liver. Lanthanide ions are ideal since they have similar effective ionic radii and coordination chemistry as that of Ca^{2+} (Evants 1990) in addition to the useful spectroscopic and magnetic properties (Brittain *et al.* 1976; Breen *et al.* 1985; Mulqueen *et al.* 1985). Further more, the use of lanthanide ions as calcium probes in the structural studies of calcium binding proteins (Matthews & Weaver 1974; Epstein *et al.* 1977) has been widely applied. The findings presented in this paper have important applications since lanthanide complexes such as Gd-DTPA has recently been applied as magnetic resonance imaging (MRI) reagents in clinical practice (Lauffer 1987) and there is the increasing concerns (Abbott 1975; Lauffer 1987; Evants 1990; Yuan *et al.* 1995, 1996a, b) on the toxicology and the interactive mechanism of lanthanide ions.

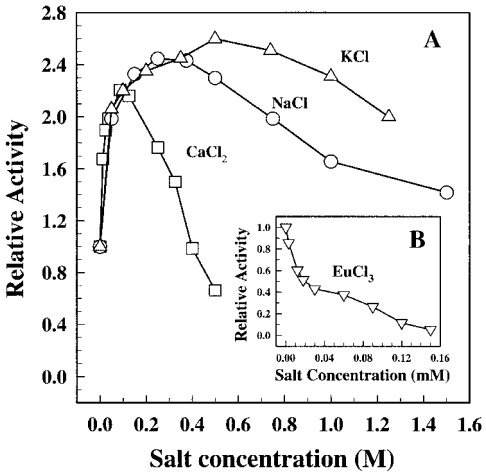

Figure 1. Effects of chlorides on chicken liver dihydrofolate reductase activity. The reaction mixture contained the following components in a total volume of 1.0 ml: 1.4 μg/ml DHFR; 50 mM Mes-NaOH pH 6.0, 10 mM β-mercaptoethanol, 0.1 mM DHF, 0.1 mM NADPH. The final concentration of salt is as indicated. The reaction was initiated by addition of DHFR for A or small volume (to avoid dilution effect) of DHF and NADPH after one hour preincubation for B.

Material and methods

Materials

Dihydrofolate (approx. 90%), NADPH (approx. 97%) and Methotrexate-agarose, MTX-Sepharose were purchased from Sigma. The concentration of DHF and NADPH was determined spectrophotometrically using molar absorption coefficient $\epsilon_{282} = 28\,000\ M^{-1}\ cm^{-1}$ (Dawson *et al.* 1969) and $\epsilon_{340} = 6200\ M^{-1}\ cm^{-1}$ (Kornberg & Horecker 1953), respectively. Sephadex G-75, IEF-Sephadex and carrier ampholine for electrofocusing were from Pharmacia. Other chemicals were of analytical grade; double-deionized water was used throughout. $EuCl_3$ solution was prepared by dissolving its oxide into HCl solution and eliminating the excess acid, and the concentration was determinated by EDTA titration using arsenazo as indicator (Yuan *et al.* 1995).

Purification of dihydrofolate reductase

DHFR from chicken liver was purified using MTX-Sepharose affinity chromatography and gel filtration (Sephadex G-75) chromatography (Kaufman 1974). DHF was removed using preparative flatbed isoelectrophoresis. The homogeneity of the final preparation was showed by a single peak in reverse-phase FPLC with a ProRPC HR5/10 column as eluted by linear gradient from 0 to 100% acetonitrile and a single band in SDS-PAGE. The specific activity of the purified enzyme was 14 μmol/min per mg. The enzyme concentration was determined by absorbance at 280 nm using a molar absorption coefficient value of $28970\ M^{-1}\ cm^{-1}$ (Kaufman 1977).

Activity measurement

The activity of DHFR was determined by following the decrease in absorbance at 340 nm at 25 °C using a Shimadzu UV-2101PC spectrophotometer (Matthews *et al.* 1963). The reaction mixture contained 50 mM Mes-NaOH buffer (pH 6.0), 10 mM β-mercaptoethanol, 0.1 mM DHF, 0.1 mM NADPH and the required concentration of salts.

Fluorescence measurements

Fluorescence measurements were performed on a Hitachi F-4010 fluorimeter at 25 °C. All experiments were performed in 50 mM Mes-NaOH buffer (pH 6.0) containing 10 mM β-mercaptoethanol. The excitation and emission slits were set at either 5 nm or 10 nm as indicated in figure legends. The scanning speed was 120 nm/min. The excitation wavelength for protein fluorescence was set at 280 nm. For Eu^{3+} emission spectrum, the excitation wavelength was 319 nm or 395 nm; while for excitation spectrum, the emission wavelength was set at 593 nm.

Results and discussion

Influence of metal chlorides on the activity of DHFR

Stimulation of the activity of chicken liver dihydrofolate reductase by different metal chlorides is illustrated in Figure 1. At low concentrations of monovalent salts, NaCl and KCl were found to stimulate DHFR activity, and reaching a maximum activation of approximately 2.5 fold at concentrations of about 0.4–0.5 M. However, at high salt concentration (> 0.6 M), the activity declines. Similar effects of these salts have been reported for DHFR from L1210 cells (Reyes & Huennkens 1967). The divalent salt, $CaCl_2$ shows a similar effect at low concentration to those of monovalent salts with maximal activation occurring at a lower concentration (0.1 M), and with a slightly lower activation ($\sim$2.2 fold). Further increasing the concentration of $CaCl_2$ leads to inactivation and even inhibition of this enzyme (> 0.4 M). The influence of metal ions on the activity of DHFR has been ascribed to their contribution to the ionic strength of solvent (Barbehenn & Kaufman 1982). If this is indeed the case then, 0.4 M $CaCl_2$ solution would be expected to have comparable ionic strength to 1.2 M NaCl. However, as it apparent in Figure 1A, 0.4 M $CaCl_2$ only produces about 1.6-fold activation. Similarly, NaCl and KCl, which have the same ionic strength, display different activation behavior. Therefore, these results suggest that the activation process of these ions cannot be simply ascribed to their ionic strength properties. Furthermore, if the activation of the enzyme is the results of the common chloride anion as described by Reyes (Reveys & Huennkens 1967), then half concentration of $CaCl_2$ would be expected to have the same effect as NaCl or KCl. This does not occur as shown in Figure 1A that the activity of the enzyme is inhibited by 0.4 M $CaCl_2$, while, at 1.6 M NaCl and KCl, the enzyme is still activated. This suggests that the activation process by these ions cannot be simply ascribed to the common chloride anion.

In Figure 1B, the effect of nanomolar concentrations of trivalent lanthanide salt is shown. In contrast with mono- and divalent salts, trivalent lanthanide salts, strikingly, do not show any detectable activation, even at the nanomolar concentration range. Figure 1B shows that $EuCl_3$ strongly inhibit the activity of DHFR, for example, the activity of DHFR declines to 50% of that control in the presence of 0.02 mM $EuCl_3$. Furthermore, the mode of activation observed for NaCl, KCl, and $CaCl_2$ is rapid (mixing time), the inhibition by $EuCl_3$ is a slow, reversible process (Tsou 1988) as indicated by gradual decrease of the assay curve until a straight line is reached. The addition of EDTA solution can fully recover the activity at Eu/EDTA molar ratio of 1:1. Clearly, the strong inhibition at such lower concentration of $EuCl_3$ cannot be due to changes in ionic strength or the presence of the common chloride anion. Rather the differences in effect of Eu^{3+} and other metal ions on the activity of DHFR suggests that $EuCl_3$ interacts with DHFR in a different manner to that of the other chlorides. Eu^{3+} may interact specifically with the active site of DHFR, while the activation or inactivation by the other chlorides, to some extent, is due to non-specific interactions. The other chlorides may act as common stabilizers or distributors to the conformation of protein (Reyes & Huennkens 1967; Duffy *et al.* 1987). The slow, reversible kinetic properties of $EuCl_3$ inhibition suggest that Eu(III) is binding to the active site of DHFR. In order to reveal the structural specific interactions of Eu^{3+} with chicken liver DHFR, protein intrinsic fluorescence and the energy transfer spectra between protein and Eu^{3+} were measured (see below).

Intrinsic fluorescence changes

The fluorescence properties of the aromatic side chains in proteins, predominantly contributed by tryptophan, provide a sensitive means for monitoring conformational changes induced by environmental changes or metal ion binding. DHFR from chicken liver has three tryptophan residues, namely Trp24, Trp57 and Trp113. Trp24 located in the loop region connecting βA to αB is one of the strictly conserved hydrophobic amino acids at the active site and contributes to the binding of NADPH (McTigue *et al.* 1992). Trp57 and Trp113 are nonconserved amino acids located in βE and αC, respectively. Their contribution to substrate binding, if any, is unknown. Therefore the fluorescence changes are likely to represent global conformational effects.

The changes of the fluorescence emission spectra of chicken liver DHFR at different concentrations of $CaCl_2$ are shown in Figure 2A. The native enzyme with no salt incubation shows an asymmetric emission band with the maximum emission wavelength at about 323 nm. In the presence of $CaCl_2$ (low concentration region) an increase in concentration results in the gradual increase of the emission intensity with a maximum intensity observed at 0.1 M $CaCl_2$. No red shift in the emission maximum was observed. This is consistent

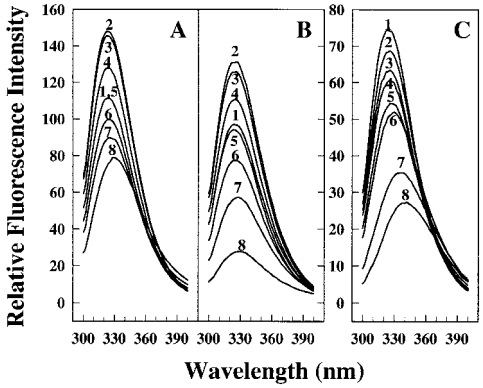

Figure 2. Fluorescence emission spectra for DHFR in the presence of (A) $CaCl_2$, (B) $CaCl_2$ and $EuCl_3$, and (C) $EuCl_3$. (A) Enzyme was preincubated with indicated concentrations of $CaCl_2$ for 2 hours. The excitation wavelength was 280 nm. Excitation and emission slits were 5 nm and 10 nm respectively. The enzyme concentration is 12.3 μg/ml. The concentrations of $CaCl_2$ for 1–8 were 0, 0.075, 0.125, 0.25, 0.5, 1.0, 1.5 and 2.0 M, respectively. (B) The same as described in (A) except that the emission spectra were recorded after adding of $EuCl_3$ (5 μl to 2000 μl system). The final concentration of $EuCl_3$ was 1.5 mM. (C) 24.6 μg/ml enzyme was preincubated with indicated concentration of $EuCl_3$ for 2 hours. Excitation and the emission slits were set both at 5 nm. The concentrations of $EuCl_3$ from 1–8 were 0, 0.15, 0.3, 0.6, 1.5, 3.0, 6.0 and 15 mM, respectively.

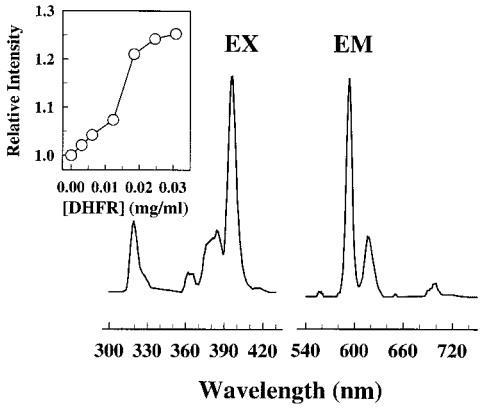

Figure 3. Excitation (EX) and emission (EM) spectra for Eu^{3+} in 50mM Mes-NaOH pH 6.0. The excitation and emission slits were set at 5 nm. The concentration of $EuCl_3$ was 0.15 mM. Inset: relative changes of the intensity at 319 nm of Eu^{3+} as a function of DHFR concentrations.

with the concentration range of the activation. Further increases in $CaCl_2$ concentration (>0.1 M) are accompanied by intensity decreases and a gradual red shift in the emission maximum (from 323 nm to 330 nm).

Figure 2C shows the changes of the fluorescence emission spectra of chicken liver DHFR at different concentrations of $EuCl_3$. The presence of $EuCl_3$ produces a dramatic decrease in emission intensity and red shifts in the emission maxima at millimolar concentration range. The presence of 0.15 mM $EuCl_3$ causes the emission intensity to decrease about 10%; when the concentration of $EuCl_3$ reaches 15mM, the emission intensity decreased to 30% of native with no salt, with a 17 nm red shift of the emission maximum. This decrease in emission intensity and pronounced red shift demonstrates a dramatic conformational change of DHFR as the result of $EuCl_3$. Nonradiative energy transfer from the side chains of the aromatic amino acids to Eu(III) may also contribute to the decrease of the intrinsic fluorescence of protein. However, in the absence of a conformational change, its quenching role will be very weak (Breen *et al.* 1985). The addition of more $EuCl_3$ increases the exposure extent of tryptophan residues to a hydrophilic environment. Comparing the conformational change with the inhibition of DHFR activity by $EuCl_3$, we found that the activity of DHFR had been inhibited to 50% of that control in the presence of 0.02 mM $EuCl_3$. However at these concentrations, the protein intrinsic fluorescence shows no detectable change in intensity and no red shift in emission maximum, indicating the inhibition process preceded the unfolding of the protein. The activity is already inhibited to some extent with no detectable change in the conformation of whole protein. This result cannot be explained in terms of the common anion (Reyes & Huennkens 1967) or ionic strength effect (Barbehenn & Kaufman 1982). A reasonable interpretation is that Eu^{3+} can specifically interact with the active site of DHFR, result in the local conformational change at the active site that may not be detected by protein intrinsic fluorescence, and somewhat interfere with the binding of substrate to the enzyme (Tsou 1993). Since the intrinsic fluorescence of DHFR comes primarily from the contribution of tryptophan residues, the interaction of Eu^{3+} with tryptophan residues, especially Trp24 is of great interest. The characteristic luminescence spectra of Eu(III) was used to provide more information on the interaction of Eu^{3+} with DHFR (see below).

Luminescence spectra of Eu(III) and energy transfer

The characteristic luminescence spectra of Eu^{3+} are illustrated in Figure 3. A strong emission peak at 593 nm is observed upon excitation at 319 nm and 395 nm, and has been attributed to the 4f–4f transitions between the ground state of Eu^{3+} ion and its excited states (Wu & Shi 1995). The relative change of excitation intensity at 319 nm as a function of DHFR

concentrations is shown in the inset of Figure 3. An increase of the DHFR concentration leads to a gradual increase of the excitation intensity at 319 nm (λem = 593 nm) indicative of energy-transfer from DHFR to Eu^{3+}. Here the tryptophan residues are the energy donor as well as the main contributor of protein intrinsic fluorescence.

We used $CaCl_2$ as an indicator to study different conformational states of DHFR. Identical concentrations of DHFR solutions were preincubated with different concentrations of $CaCl_2$ and allowed to fully equilibrate. After recording the initial conformational state of protein (Figure 2A), small volume of $EuCl_3$ (to avoid dilution effect) is added to each reaction system for fluorescence measurement.

In Figure 2B, a remarkable difference is observed between the intrinsic fluorescence quenching of protein in different conformational states by the same concentration of $EuCl_3$. In Figure 4A, show that, increase of $CaCl_2$ concentration enhances the degree of conformational extension and the extent of exposure of its fluorophore (tryptophan) to the hydrophilic environment. At the same time, the 319 nm excitation intensity ratios, I_{319}/I^0_{319} of Eu(III) are also increased (Figure 4B) indicating intensified energy transfer efficiency. Therefore, Eu(III) can play two roles in contributing to the dramatic quenching observed for DHFR protein intrinsic fluorescence; one is as conformational inductor and the other as an energy acceptor. The two functions are cooperative, with the more open conformation of protein having a more solvent exposure of the tryptophan and hence the higher efficiency of the energy transfer.

The efficiency of energy transfer E, in dipole-dipole nonradiative energy transfer, between a donor and acceptor is related to the spatial distance of separation, r, and the critical distance for 50% energy transfer, R_0, is given by the following equation: $E = [1 + (r/R_0)^6]^{-1}$ (Förster 1967). So the observed increase in energy transfer efficiency during conformational extension of protein indicates an increased access of Eu^{3+} to the fluorescent tryptophan core. The crystal structure of DHFR•$NADP^+$•biopterin ternary complex (McTigue *et al.* 1992) shows Trp57 and Trp113, are located in βE and αC, respectively, which is close to the center of the protein molecule. The distances between these two tryptophans and the Eu(III) ion in solvent are estimated to be at least 20 Å. The contribution of these two residues to energy transfer must be weaker compared to the solvent exposed donor. However Trp24 located in the mobile loop region connecting βA to αB, is more accessible to solvent, so the low energy transfer efficiency from DHFR at slight conformational change states (corresponding to the region while the concentration of $CaCl_2$ is less than 0.5 M), is most likely contributed by this tryptophan residue. Only when the $CaCl_2$ concentration is greater than 0.5 M, and the whole conformation of DHFR dramatically extends is Eu(III) accessible to the other two tryptophan residues and therefore leads to the highly efficient energy transfer.

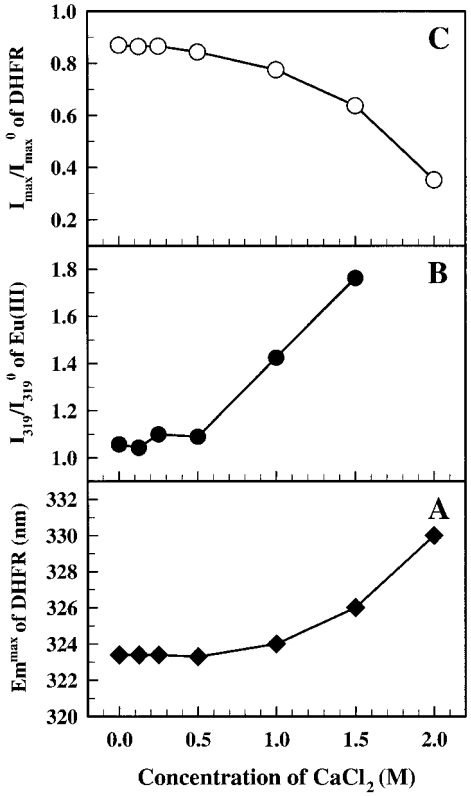

Figure 4. The interaction between $EuCl_3$ and DHFR at the different conformation state induced by $CaCl_2$. The enzyme concentration is 12.3 μg/ml and the final concentration of $EuCl_3$ is 1.5 mM. For protein intrinsic fluorescence measurement, the excitation wavelength was 280 nm, excitation and emission slits were 5 nm and 10 nm, respectively. While for Eu(III), the excitation and emission slits were set both at 5 nm. Its excitation spectrum was measured by fixing the monitoring wavelength at 593 nm. (A) The red shift in emission wavelength maxima of DHFR as a function of $CaCl_2$ concentrations. (B) The relative changes of Eu(III) 319 nm excitation intensities after $EuCl_3$ added to DHFR solutions containing different concentrations of $CaCl_2$as conformational inductor. (C) The relative changes of DHFR maximal emission intensities after adding $EuCl_3$.

Inhibition mechanism of Eu(III)

Dihydrofolate reductase (DHFR) can be activated by mono- and bivalent salts (Reyes & Huennkens 1967; Barbehenn & Kaufman 1982) and the effects of these metal salts have been ascribed to the common anion (Reyes & Huennkens 1967) and ionic strength (Barbehenn & Kaufman 1982). The role of cations was not clarified. The different behavior between NaCl and $CaCl_2$, and even NaCl and KCl on the activation of DHFR from L1210 cells is apparent, which is not satisfactorily accounted for by either explanation. The original motive for this study was to use Eu(III) as fluorescent probe to study the ion-dependent activation of DHFR. However, surprisingly $EuCl_3$ shows a strong inhibition of DHFR activity even at very low concentration. The slow, reversible inhibition kinetics indicates the binding of Eu(III) to the active site of DHFR. At the same time, the energy transfer experiments suggest the low energy transfer efficiency obtained in the more compact state of protein is due to the Trp24→Eu(III) interaction. Therefore the inhibition of lanthanide ion on the activity of DHFR may be due to the specific interaction of Eu^{3+} with Trp24 residue, a strictly conserved at the active site for chicken liver DHFR. The crystal structure data (McTigue *et al.* 1992) provide additional support for this interpretation. The water-230 in the crystal structure, as the terminal water in one of important solvent channels, is linked through hydrogen bonds to the side-chain carboxylate of Glu30, the indole of Trp24 and O4 of biopterin and plays an important role for proton transfer to pteridine. This functional region is linked with the mobile loop connecting βA to αB, which contributes to the transition-state stabilization (Li *et al.* 1992). It is probable that lanthanide ions can pass through this solvent channel and hence interact with Trp24 residue. This process would be expected to be slow and the binding lanthanide ions are in equilibrium with bulk ions. The different effects for Ca^{2+} and Eu^{3+} may due to the stronger ability of Eu^{3+} to combine oxygen and nitrogen atoms in amino acids, therefore at low concentration, Eu^{3+} can bind to Trp24, and prevent the substrate binding. However, Ca^{2+}, is inhibitory only at high concentration (Figure 1). The ability of Na^+ and K^+ to combine with the amino acid is very weak, so they show no inhibition of DHFR activity even at high concentration, therefore the activation of these salts is mainly due to ionic strength effect. Further investigations to elucidate the kinetic mechanism for Eu(III) inhibition are in progress.

Conclusions

In order to understand the mechanism of ion-dependent activation of Dihydrofolate reductase (DHFR) from chicken liver, we have studied the effects of mono-, bi- and trivalent metal ions (served as chlorides) on the activity of this enzyme. The monovalent metal ions (Na^+, K^+) can activate this enzyme by 2.5-fold at 0.4–0.5 M. Ca^{2+} shows similar activation effect as that of monovalent ions, although at high concentration, Ca^{2+} is inhibitory. Lanthanide ions, surprisingly, show no activation but inhibition on the activity of this enzyme even at very low concentration. This inhibition is a slow, reversible process. Luminescence spectra of Eu(III) and energy transfer measurements show that Eu^{3+} can specifically interact with Trp24 residue, which is located at active site of this enzyme, and thereby inhibits the activity of this enzyme.

Acknowledgements

Part of the work was carried out in Institute of Biophysics, Chinese Academy of Science. I like to thank Dr. Louise Wallace for help in preparation of this manuscript and Dr. Chunbo Yuan from Steacie Institute, NRC, Canada for many helpful discussions.

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