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Hydrophobic interactions in biological systems: some background information based on ligand–ligand interactions in metal ion complexes

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Abstract - In mixed ligand M(ATP)(L) complexes, where L = 2,2'-bipyridyl (Bpy), tryptophanate (Trp^-) or leucinate (Leu^-), intramolecular stacks and hydrophobic adducts form between the purine moiety of ATP^{4-} and the aromatic-ring or alkyl residues of L. Corresponding intramolecular equilibria occur in M(Phen)(L) complexes (M = Cu^{2+} or Zn^{2+}, Phen = 1,10-phenanthroline, and L = phenylalkane- or isoalkane-carboxylates) and these allow studies on the influence of the solvent composition: addition of some ethanol (or dioxane) to an aqueous solution favors formation of the intramolecular adducts. This result is in contrast to the experience with simple unbridged stacks or hydrophobic adducts, which are destabilized. Based on these observations a 'mechanism' for cooperative effects regarding substrate binding in the active-site cavity of metalloenzymes is proposed. In binary $M(AA)_2$ and ternary M(AA)(AA') complexes, where AA and AA' = Trp^-, Leu^-, etc., also intramolecular adducts are formed.

1. INTRAMOLECULAR STACKS AND HYDROPHOBIC ADDUCTS IN MIXED LIGAND COMPLEXES

The discovery in 1974 of intramolecular stacking interactions between the purine moiety of adenosine 5'-triphosphate (ATP^{4-}) and the aromatic rings of 2,2'-bipyridyl (Bpy) in mixed ligand $M(Bpy)(ATP)^{2-}$ complexes (Fig. 1) [1][a] has added a new 'dimension' regarding the variables for the stability and structure of complexes in solution [2]. The formation degree of intramolecular stacks in such systems containing nucleotides is high [2-4]; stack formation is evident from an increased complex stability [5] and confirmed by the appearance of UV charge-transfer bands [1,5,6] and by the observation of upfield shifts in the ^1H NMR spectra [5,7,8], as well as for the solid state by X-ray crystal studies [9-11].

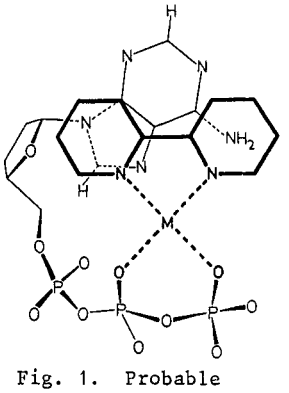
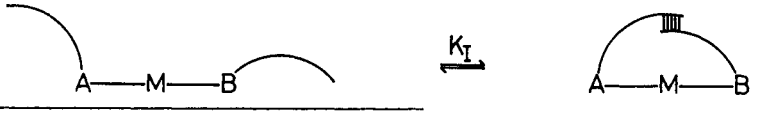


Fig. 1. Probable (schematic) structure of the species with an intramolecular stack for $M(Bpy)(ATP)^{2-}$ in solution.

Many investigations on biologically more relevant systems have lately appeared [12-15] showing, e.g., that $M(ATP)(Trp)^{3-}$ and $M(ATP)(Leu)^{3-}$ complexes form intramolecular adducts between the purine moiety of ATP^{4-} and the indole side-chain of tryptophanate (Trp^-) [16] or the isopropyl residue of leucinate (Leu^-) [16,17]. Some of the results are summarized in Fig. 2; the occurrence of purine-indole stacks in $M(ATP)(Trp)^{3-}$ species has been repeatedly confirmed [18-21]. The structure and stability of mixed ligand complexes formed between $M(ATP)^{2-}$ and an amino acid anion varies; the affinity for $M(ATP)^{2-}$ decreases in the series, Trp^- > Leu^- > Ala^- (Fig. 2). Regarding the origin of the genetic code [22] and the recognition interactions between nucleic acids (or nucleotides) and proteins (or amino acids) [23] such observations deserve notice.

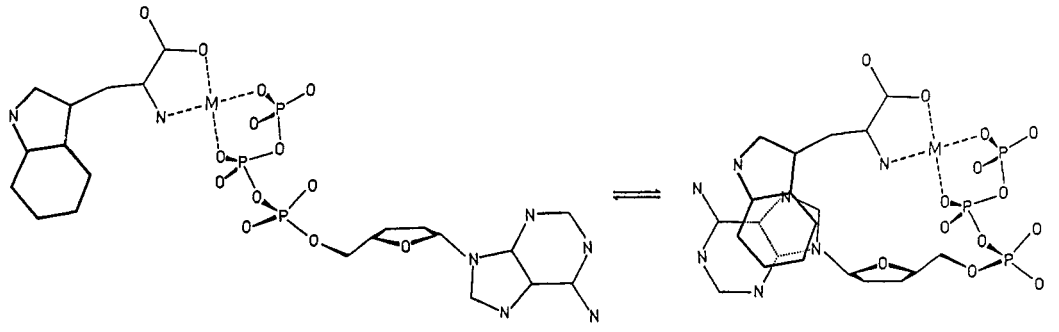
The position of intramolecular equilibria as depicted in Fig. 2 and as expressed in a general way in equilibrium 1 may be calculated from the stability constants of the complexes as deter-



(1)

a) The numbers of the references are given in square brackets and those of equilibria and reactions in parentheses.

Abbreviations: A and B, symbols for two different ligands; CA^-, carboxylate ligand; CPA, carboxypeptidase A; M, general metal ion; PPr^-, 3-phenylpropionate; for further definitions see text and figures.



Approximate percentages of the 'closed' (cl) species in aqueous solution for

	% $M(ATP)(Trp)^{3-}_{cl}$			% $M(ATP)(Leu)^{3-}_{cl}$	
Mn^{2+}	52	[17]	Mn^{2+}	41	[17]
Cu^{2+}	35	[17]	Cu^{2+}	25	[17]
Zn^{2+}	74 (40±15)	[8,17]	Zn^{2+}	~5 (~30/20,75)	[17]
Mg^{2+}	(44±19)	[8]	Cd^{2+}	(~10/ 5,25)	[17]

Fig. 2. Intramolecular equilibrium for $M(ATP)(Trp)^{3-}$ complexes and formation degree of the closed species; for comparison the data for the corresponding ternary complexes with leucinate (Leu^-) are also listed. The percentages given in parentheses are from ^1H NMR upfield shift measurements in D_2O (the error ranges or the lower and upper limits are estimated); the other results are based on stability constants determined by potentiometric pH titrations (I = 0.1 M, $NaClO_4$; 25°C).

mined by potentiometric pH titrations. If the two isomeric forms are designated as 'open' and 'closed', i.e. $M(A)(B)_{op}$ and $M(A)(B)_{cl}$, respectively, the dimensionless constant of equilibrium 1 is defined by eq. 2:

$$K_I = [M(A)(B)_{cl}]/[M(A)(B)_{op}] \tag{2}$$

Values of K_I may be calculated from eq. 3 [2,13,24],

$$K_I = \frac{K_{exp}}{K_{op}} - 1 = \frac{10^{\Delta \log K_{(M/A/B)}}}{10^{\Delta \log K_{(M/A/B)_{op}}}} - 1 = 10^{\Delta\Delta \log K} - 1 \tag{3}$$

which involves the following definitions:

$$M(A) + B \rightleftharpoons M(A)(B)_{op} \rightleftharpoons M(A)(B)_{cl} \tag{4a}$$

$$K^{M(A)}_{M(A)(B)} = K_{exp} = [M(A)(B)]/([M(A)][B]) = ([M(A)(B)_{op}]+[M(A)(B)_{cl}])/([M(A)][B]) \tag{4b}$$

$$K^{M(A)}_{M(A)(B)_{op}} = K_{op} = [M(A)(B)_{op}]/([M(A)][B]) \tag{5}$$

$$\Delta \log K_{(M/A/B)} = \log K^{M(A)}_{M(A)(B)} - \log K^{M}_{M(B)} = \log K^{M(B)}_{M(B)(A)} - \log K^{M}_{M(A)} \tag{6a}$$

$$M(A) + M(B) \rightleftharpoons M(A)(B) + M \tag{6b}$$

$$\Delta \log K_{(M/A/B)_{op}} = \log K^{M(A)}_{M(A)(B)_{op}} - \log K^{M}_{M(B)} = \log K^{M(B)}_{M(B)(A)_{op}} - \log K^{M}_{M(A)} \tag{7}$$

$$M + B \rightleftharpoons M(B) \qquad K^{M}_{M(B)} = [M(B)]/([M][B]) \tag{8}$$

$$\Delta\Delta \log K = \Delta \log K_{(M/A/B)} - \Delta \log K_{(M/A/B)_{op}} \tag{9}$$

Two points should be noted: (i) The difficulty usually is to obtain reliable values for the open species, i.e. for $\log K_{op}$ (eq. 5) or $\Delta \log K_{(M/A/B)_{op}}$ (eq. 7). (ii) Any error in the experimentally determined constants will be the more significant, the smaller the difference is in equation 9; well defined error limits of all constants are therefore compulsory.

2. DECREASING SOLVENT POLARITY AND COMPLEX STABILITY

It is now generally agreed that different types of water exist in cells [25], variously described as bound water, structured water regions, bulk water, etc.. Closely connected herewith is a change of the solvent polarity on the surface of proteins [26] or in the active-site cavities of enzymes [27]. The so-called equivalent solution dielectric constants in the

active-site cavities of carbonic anhydrase and carboxypeptidase A were estimated as 35 and 70, respectively [27]. For cytochrome c_{551} an effective dielectric constant of 27 is given for the region between the heme iron and a propionate residue; the charged groups are buried at least 5 Å beneath the protein surface [28]. For a similar situation in ferricytochrome c an effective dielectric constant of about 50 has been estimated [29].

It is evident that a change of polarity in the micro-environment of a reactive site can have a drastic influence, but our knowledge in this respect is still very limited. The acidity constants of H^+(N) and H-O sites are clearly dependent upon the polarity of the solvent [30]: the basicity of uncharged N sites usually decreases as the polarity of the solvent decreases, while the basicity of negatively charged O^- sites increases markedly as the dielectric constant decreases. The changes in complex stability are in accord with the basicity changes [30]: with N ligands the stability decreases usually only slightly with decreasing polarity of the solvent, while with O ligands [27] the increase in stability may be quite pronounced.

In the present context a study [31] dealing with the Cu^{2+}/ATP/1,10-phenanthroline (Phen) system is of special interest, because also the influence of increasing amounts of dioxane on the intramolecular equilibrium between $Cu(Phen)(ATP)^{2-}_{op}$ and $Cu(Phen)(ATP)^{2-}_{cl}$ (eq. 1) was investigated, together with the stability of the unbridged binary $(Phen)(ATP)^{4-}$ stack under the corresponding conditions. It is revealing to consider the formation degrees of the stacks for 10^{-3} M reactant solutions (I = 0.1 M, $NaNO_3$; ~25°C; pH ~7) [31] under the following conditions:

Solvent	% $(Phen)(ATP)^{4-}$	% $Cu(Phen)(ATP)^{2-}_{cl}$
water	3.5	90
50% (v/v) dioxane-water	0.18	46

At least two facts are obvious: (i) The presence of the organic solvent inhibits stacking in both systems, but for the unbridged stack the inhibition factor is about 1/20 while for the ternary stack it is only 1/2. (ii) The formation of a metal-ion bridge between the individual parts of a stacking adduct favors the stability of this adduct strongly; the promotion factor in water is about 25 while in the dioxane-water mixture it is about 250.

The preceding results indicate that selectivity is much more pronounced under conditions with a reduced solvent polarity [32], despite the lower formation degrees of the stacks, because the effect of the bridging metal ion is most dramatic. This observation is meaningful for biological systems and warrants further studies; some are summarized in the next section. One of the difficulties in working with aqueous-organic solvent mixtures is the solubility of the ligands and their metal ion complexes; this has forced us to employ the ligands of Figure 3 which appear at first sight as biologically less meaningful.

3. TERNARY COMPLEXES CONTAINING 1,10-PHENANTHROLINE AND PHENYLALKANE- OR ISOALKANE-CARBOXYLATES – SOLVENT INFLUENCE

To learn more about the factors that govern and determine the extent of intramolecular hydrophobic or stacking interactions the mixed-ligand systems shown in Fig. 3 were studied [33-36].

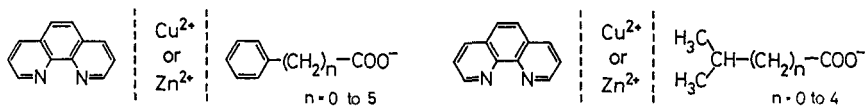


Fig. 3. Mixed ligand systems and metal ions considered in Section 3.

The indicated series of carboxylate ligands (CA^-) allows a systematic variation of the distance between the phenyl ring or the isopropyl moiety undergoing adduct formation with Phen and the coordinating carboxylate group, as well as of the overall size of the alkyl residue. Both properties, distance and overall size, are expected to influence the extent of the interaction. Similarly, the geometry of the coordination sphere of the bridging metal ion is also expected to have an influence: Cu^{2+} has a tetragonal coordination sphere allowing four equatorial and nearby donor atoms and possibly one or two more distant axial donors, while Zn^{2+} has a tetrahedral or octahedral geometry. Indeed, these differences are reflected in the stability of the complexes [34,36], especially in those that involve short chain alkanecarboxylates [36] because differences in the overlapping area (eq. 1) affect the stabilities of the adducts. Only some of the results [33-36] are indicated in the remainder of this section.

The data given in Table 1 show that the number of methylene groups between the phenyl ring and the coordinating carboxylate affects the stability of the intramolecular stack, which reaches its largest formation degree in 50% (v/v) dioxane-water with $Cu(Phen)(2\text{-phenylacetate})^+$ (Fig. 4). Replacement of the phenyl ring by the larger naphthyl moiety favors stacking, as one

TABLE 1. Extent of intramolecular aromatic-ring stacking in ternary Cu(Phen)$(CA)^+$ complexes [34], where $CA^- = C_6H_5-(CH_2)_n-COO^-$ with n = 0 to 5, in 50% (v/v) dioxane-water (mol fraction of dioxane 0.175; dielectric constant ε = 35.2 [37]) at 25°C and I = 0.1 M ($NaClO_4$)[a]

CA^-	$\Delta \log K_{(Cu/Phen/CA)}$ (eq. 6)	$\Delta\Delta \log K$ (eq. 9)[b]	K_I (eq. 2,3)	% $Cu(Phen)(CA)^+_{cl}$ (eq. 1)
n = 0	0.14 ± 0.03	0.10 ± 0.03	0.26 ± 0.10	21 ± 6
1	0.46 ± 0.04	0.42 ± 0.05	1.63 ± 0.29	62 ± 4
2	0.28 ± 0.02	0.24 ± 0.03	0.74 ± 0.11	43 ± 4
3	0.20 ± 0.03	0.16 ± 0.03	0.45 ± 0.11	31 ± 5
4	0.22 ± 0.01	0.18 ± 0.02	0.51 ± 0.08	34 ± 3
5	0.20 ± 0.02	0.16 ± 0.03	0.45 ± 0.10	31 ± 5
β-NAc^- [c]	0.70 ± 0.04	0.66 ± 0.04	3.57 ± 0.42	78 ± 2

a) The errors given are *three times* the standard error of the mean value [34].
b) Calculated with $\Delta \log K_{Cu/Phen/CA)_{op}} = 0.04 \pm 0.02 \approx 1/2(\Delta \log K_{(Cu/Phen/HCOO)} + \Delta \log K_{(Cu/Phen/Ac)}) = 1/2[(0.03 \pm 0.03) + (0.04 \pm 0.02)]$.
c) β-NAc^- = 2-(β-naphthyl)acetate.

might expect, and in 50% dioxane-water a formation degree of nearly 80% is reached with Cu(Phen)[2-(β-naphthyl)acetate]$^+$. The indole residue exhibits similar stacking properties, and with regard to amino-acid side-chains it is interesting to note that the tendency to form intramolecular stacks in such ternary complexes decreases in the series, indole > phenyl > imidazole (≙ pyrrole derivative) [34]. The formation degree of the intramolecular stack for Cu(Phen)(2-phenylacetate)$^+$ in water reaches only 48 (±9)% [34], i.e. stack formation is more pronounced in 50% dioxane-water (Table 1 and vide infra). This result should be compared with the effect of dioxane on the stability of the unbridged stack formed between $Zn(Phen)^{2+}$ and benzyl alcohol: K = 2.2 ± 0.2 M^{-1} in water [33] and 0.49 ± 0.09 M^{-1} in 50% (v/v) dioxane-water [35].

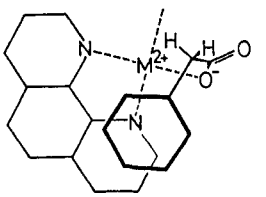


Fig. 4. Probable (schematic) structure of the species with an intramolecular stack for M(Phen)(2-phenylacetate)$^+$ in solution.

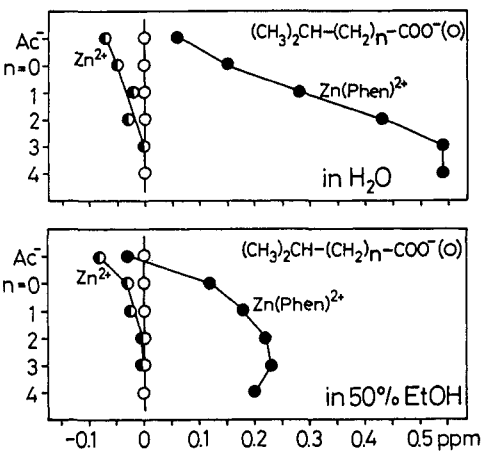


Fig. 5. Up- and downfield shifts of the terminal methyl group(s) in the ^1H NMR spectra of acetate (Ac^-) and isoalkanecarboxylates for Zn^{2+} (◐) or $Zn(Phen)^{2+}$ (●) coordination in water (upper part) and 50% (v/v) ethanol-water (lower part) relative to the resonance positions of the uncoordinated carboxylates (○). The measurements were carried out at I = 0.1 - 0.2 M ($NaNO_3$) and 34°C. Reproduced by permission of the American Chemical Society from ref. [36].

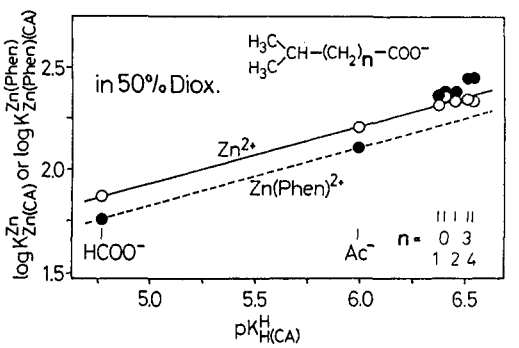


Fig. 6. Relationship between log $K^{Zn}_{Zn(CA)}$ or log $K^{Zn(Phen)}_{Zn(Phen)(CA)}$ and $pK^H_{H(CA)}$ in 50% (v/v) dioxane-water for the binary complexes, $Zn(CA)^+$ (○), or the ternary complexes, $Zn(Phen)(CA)^+$ (●), with *simple* carboxylates, i.e. $HCOO^-$ and CH_3COO^- (= Ac^-), and the isoalkanecarboxylates (see formula above) with n = 0 to 4. The plotted equilibrium constants are from ref. [36], and those for the binary complexes fit on a straight line (*solid* line; regression: m = 0.283 ± 0.012, y_o = 0.519 ± 0.075, 1σ); the reference line for the ternary complexes (*broken* line) is drawn with the corresponding slope (m) but only through the points of $HCOO^-$ and Ac^-.

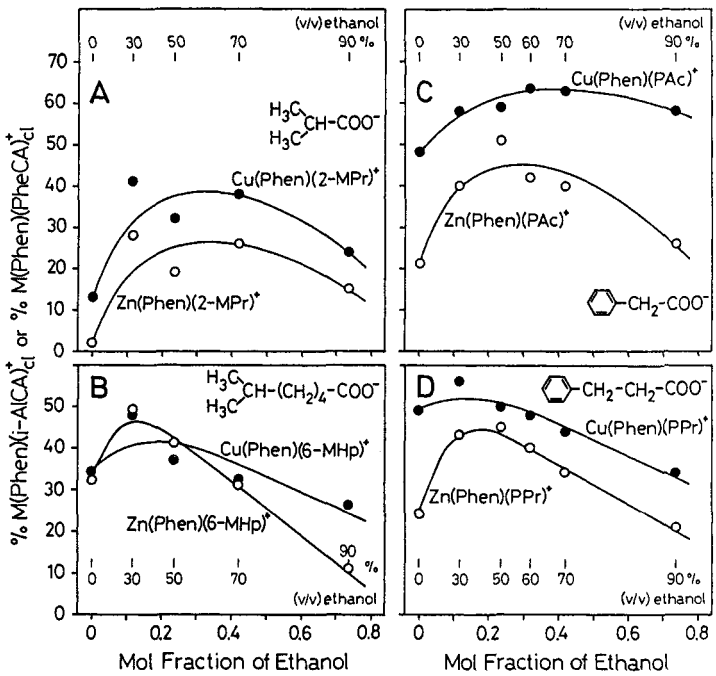


Fig. 7. Formation degree of the intramolecularly closed species (eq. 1) in ternary M(Phen)(2-methylpropionate)$^+$ (A), M(Phen)(6-methylheptanoate)$^+$ (B), M(Phen)(2-phenylacetate)$^+$ (C), and M(Phen)(3-phenylpropionate)$^+$ (D) complexes of Cu^{2+} (●) and Zn^{2+} (○) depending on the mol fractions of ethanol; the second solvent component is always water (I = 0.1 M; 25°C). The dielectric constants (in parentheses) are for water (78.5), 30% ethanol (63.7), 50%, (52.1), 60% (46.1), 70% (40.8), and 90% ethanol (29.1); these values are interpolated from the data given in ref. [38]. Reproduced by permission of the American Chemical Society from ref. [36].

Evidently one may expect that with increasing distance between the adduct forming moiety and the coordinating carboxylate group a situation is approached in which the adduct forming moiety reaches beyond the phenanthroline ring system (cf. with Fig. 4). Indeed, by ^1H NMR shift studies with the isoalkanecarboxylate ligands and $Zn(Phen)^{2+}$ this may be shown [36]: the maximum upfield shift is reached with 5-methylhexanoate (n = 3) as is seen in Fig. 5. It is further obvious that the downfield shifts occurring upon Zn^{2+} coordination are comparable in both solvents, while the upfield shifts resulting from $Zn(Phen)^{2+}$ coordination are more pronounced in water than in 50% (v/v) ethanol-water; this may indicate that the structure of the intramolecular adducts is somewhat different in the two solvents (vide infra). However, that in an aqueous-organic solvent mixture still a significant stability increase for the ternary complexes with an intramolecular ligand-ligand interaction occurs is evident from Fig. 6: the data points for the $Zn(Phen)^{2+}$ complexes with the isoalkanecarboxylates are clearly above the reference line (broken line), i.e. these ternary complexes are more stable by about 0.1 to 0.2 log unit (which corresponds to $\Delta\Delta$ log K; eq. 9) than expected on the basis of the basicity of the coordinated carboxylate groups [36].

The effect of an organic solvent added to an aqueous solution on the formation degree of an intramolecular adduct (eq. 1) is best seen in Fig. 7, where the percentages of the closed species (eq. 1) in dependence on the mol fractions of ethanol are plotted for the ternary M(Phen)(CA)$^+$ complexes of two isoalkanecarboxylates (i-AlCA$^-$; left parts) and two phenylalkanecarboxylates (PheCA$^-$; right parts) [36]. The influence of dioxane on the formation degree of the adducts in M(Phen)(PheCA)$^+$ complexes is very similar to that of ethanol [35]. Addition of (some) ethanol or dioxane to an aqueous solution of these ternary complexes *favors* the formation degree of the closed species. Only in solutions that contain more than about 70% ethanol (Fig. 7) does the concentration of the closed species decrease, though it may still be of the order found in aqueous solution. This observation is clearly contrary to the experience with simple *unbridged* hydrophobic or stacking adducts [35,36].

There are indications (e.g., Fig. 5) [35,36] that the structure of the closed species is solvent-dependent: in water, a 'simple' (though not rigid) intramolecular ligand-ligand adduct is formed, while in the mixed solvents, in addition, probably a series of structurally somewhat different closed species (orientation of the ligand moieties, degrees of solvation, intercalated organic solvent molecules, etc.) may occur. As there is at present no way to identify with certainty such different structures, the whole observed stability increase between M(Phen)(PheCA)$^+$ or M(Phen)(i-AlCA)$^+$ and M(Phen)(Acetate)$^+$ or M(Phen)(HCOO)$^+$ is simply attributed to a (single) so-called "closed" species (Table 1; Fig. 7) [35,36]. It should be noted that the stability of the M^{2+}/O^- interaction (eq. 4,5,8) is governed by the polarity of the solvent [27,35,36], while the position of the intramolecular equilibrium 1 is influenced by the hydrophobic solvation properties of the organic solvent molecules.

Possible explanations for these observations (Fig. 7) have been discussed in detail [35,36]. In short, it appears that addition of small amounts of an organic solvent to an aqueous solution of the complexes leads to a preferred hydrophobic solvation of the intramolecular stack or hydrophobic adduct (eq. 1; Fig. 4) because the lypophilicity of this adduct acts as a germ and attracts the organic molecules forming a micelle-like hydrophobic unit close to the metal ion. The formation of this "micelle" will favor the closed species and it may also reduce the *effective* dielectric constant close to the metal ion and consequently the M^{2+}/O^- binding [27] would be stabilized; in fact, this would be quite a powerful mechanism [35]. It is evident, that as soon as this micelle-like unit has reached a size where it is coming into conflict with the hydration sphere of the bridging metal ion, no further organic solvent molecules can favorably be added [35,36]. Now, with increasing and already relatively large amounts of organic solvent molecules, the hydrophobic residues of the open isomer in equilibrium 1 are individually solvated.

4. A HYPOTHESIS: ARE THERE COOPERATIVE EFFECTS BETWEEN HYDROPHOBIC AND POLAR INTERACTIONS? A CONSIDERATION ON ACTIVE SITE CAVITIES OF ENZYMES

The observations summarized in Section 3 and culminating in the results of Figure 7 [35,36] may be used to interpret the binding of substrates in active-site cavities of enzymes. The corresponding view will be illustrated with the Zn^{2+} metalloenzyme carboxypeptidase A (CPA) as an example. However, the outlined view is rather independent of the way in which substrates (and inhibitors) actually bind to CPA; presently a field of active research [39-41]. CPA and the available equilibrium constants merely serve to illustrate the hypothesis [35].

Acetate (Ac^-) binds to bovine CPA with a stability constant, $\log K^{(CPA-Zn)}_{(CPA-Zn)(Ac)} = 1.3$ ($I = 0.2$ M; 25°C [42]), which is only slightly larger than the binding constant measured for Zn(acetate)$^+$ in aqueous solution; $\log K^{Zn}_{Zn(Ac)} = 1.11 \pm 0.02$ ($I = 0.1$ M; 25°C [35]). The stability increase of 0.2 log unit corresponds to the slightly reduced polarity in the active-site cavity of CPA [27,35], i.e. with an *effective* dielectric constant of about 70 compared to that of bulk water ($\varepsilon = 78.5$), and this is consistent with the relatively open cavity of CPA designed to handle peptide substrates. 3-Phenylpropionate (PPr^-) and related carboxylate ligands are excellent inhibitors of CPA [41,42], and as the stability constant of Zn(PPr)$^+$ in aqueous solution is known, $\log K^{Zn}_{Zn(PPr)} = 1.14 \pm 0.04$ ($I = 0.1$ M; 25°C [35]), one may estimate the stability of the (CPA-Zn)(PPr) complex by taking into account the mentioned stability increase of 0.2 log unit: i.e., $\log K^{(CPA-Zn)}_{(CPA-Zn)(PPr)/expected} = 1.14 + 0.2 \simeq 1.34$. However, the measured constant, $\log K^{(CPA-Zn)}_{(CPA-Zn)(PPr)} = 3.77$ ($I = 0.2$ M; 25°C [42]), is about 2.4 log units *larger* than the expected one, and this indicates that with PPr^- additional interactions (not available for Ac^-) must be responsible for the stability increase. Indeed, it was suggested earlier [41,42] that aliphatic or aromatic residues of carboxylates, like the phenyl moiety of PPr^-, can conveniently be accomodated in a hydrophobic pocket in the active-site region.

However, to explain a stability increase of 2.4 log units seems hardly possible in this simple way: In the most favored cases the stability increase due to stacking for PPr^- [with $Zn(Phen)^{2+}$ or $Cu(Phen)^{2+}$] corresponds only to 0.4 log unit [35]. Certainly, this value only quantifies a one-sided interaction, but let us (be generous and) assume for an interaction in a two-sided pocket an additional factor of 10 (= 1 log unit), and then we have left unexplained still a stability increase of about 1.0 log unit for the binding of PPr^- to CPA. The suggested explanation for this additional increase in stability is a 'cooperative mechanism' which functions in the following way [35]: (i) The carboxylate group coordinates to the intrinsic Zn^{2+} of CPA, and (ii) the 'bulky' residue (of PPr^-) undergoes a hydrophobic or stacking interaction with the closeby hydrophobic pocket. (iii) The result of this is that the *effective* dielectric constant in the active-site cavity decreases, and (iv) this in turn then strengthens the polar carboxylate/Zn^{2+} interaction. Indeed, a reduction of the effective or 'equivalent solution' dielectric constant from about 70 to about 40 would lead to a stability increase of about 1.0 log unit for the Zn^{2+}/O^- interaction.

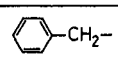
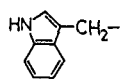
It may be pointed out in addition that the simultaneous interactions of the carboxylate group (with Zn^{2+}) and the 'bulky' hydrophobic residue (with the hydrophobic pocket) of the inhibitor (the above points 'i' and 'ii') may lead to a slight 'moving together' of the interacting sites in the enzyme cavity. In fact, the Zn^{2+} in CPA is in a movable state: binding of the 2-benzyl-3-formylpropanoate inhibitor leads to a displacement of Zn^{2+} by about 0.4 Å [43]. It is evident that such a flexibility would be ideal for metalloenzymes which catalyze the transfer of polar groups (e.g., of phosphate residues), because the release of the products from the active site would automatically be initiated as soon as the link between the polar group and the hydrophobic site is broken; the distance between them would (at least slightly) increase and the cooperativity disappear. In this way the active site would become accessible for another substrate molecule, and a continuous turnover would be guaranteed.

The indicated hypothesis about the cooperative effects between hydrophobic and polar groups includes selectivity processes and it is able to explain the relatively high stability of many metalloenzyme/substrate or inhibitor complexes, and also to rationalize the turnover processes. However, this 'cooperative mechanism' is not restricted to metalloenzymes; it may also be applied to other related interactions. For example, interrelations between hydrogen-bonding and hydrophobic interactions were recently suggested [44] for adducts formed by peptides and the glycopeptide antibiotics, vancomycin and ristocetin. It was concluded [44] "that addition of a hydrophobic group not only allows hydrophobic bonding but also strengthens existing hydrogen bonds" and that the "increased hydrogen bond strength can be an important factor in determining the overall binding energy".

5. INTRAMOLECULAR SIDE-CHAIN ADDUCTS IN COMPLEXES OF AMINO ACIDS

Hydrophobic pockets in enzymes are created by aliphatic and/or aromatic side chains of the amino acids forming the protein. Hence, one may expect that these side chains are also interacting if the amino acid anions (AA^-) are bound to metal ions; indeed, examples for intramolecular adduct formation exist in binary [45] and ternary [13,14] complexes. No reliable information is so far available in the literature on the influence of a decreasing solvent polarity on the extent of adduct formation. Therefore, in this section some recent results [46,47] are summarized on the influence of dioxane on the intramolecular adducts formed in binary $Cu(AA)_2$ complexes of the amino acids listed in Fig. 8. It should be pointed out that the side-chain residues of two amino acids with the same chirality can get in contact with each other in a $Cu(AA)_2$ complex only, if the two glycinate-like units bind in a *trans* arrangement to the equatorial part of the Cu^{2+} coordination sphere as is indicated in the lower part of Fig. 8.

L-AMINO ACIDS

$R-CH(NH_3^+)COO^-$	AA	R^-
Phenylalanine	Phe	
Tryptophan	Trp	
Alanine	Ala	CH_3-
Leucine	Leu	$(CH_3)_2CH-CH_2-$

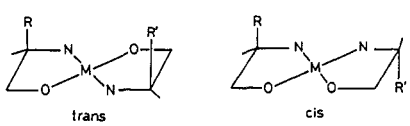


Fig. 8. Amino acids considered in Section 5, together with the possible arrangements of two amino acid anions in the equatorial part of the coordination sphere of Cu^{2+}.

The evaluations are based on the stability differences measured for the complexes formed with alaninate and those with amino acid anions having a bulky side chain. In $Cu(Ala)_2$ the side chains are too short to reach each other giving only an open form (eq. 1), the Cu^{2+}/Ala^- system is therefore a suitable reference. As an example, the results [47] are listed in Tables 2 and 3 for the Cu^{2+}/Trp system: Evidently of $Cu(Trp)_2$ significant amounts exist with an intramolecular stack, even in 80%

TABLE 2. Negative logarithms of the acidity constants of *L*-tryptophan, i.e. of $H_2(Trp)^+$, and logarithms of the stability constants of the corresponding binary $Cu(Trp)^+$ and $Cu(Trp)_2$ complexes, together with the stability differences $\Delta \log K^*_{(Cu/Trp)}$, in dependence on the amount of dioxane added to water at I = 0.1 M ($NaNO_3$), 25°C.[a] The $\Delta \log K^*_{(Cu/Ala)}$ values of the Cu^{2+}/L-alaninate system are given for comparison

% (v/v) dioxane	$pK^H_{H_2(Trp)}$	$pK^H_{H(Trp)}$	$\log K^{Cu}_{Cu(Trp)}$ (eq. 8)	$\log K^{Cu(Trp)}_{Cu(Trp)_2}$	$\Delta\log K^*_{(Cu/Trp)}$ (b)	$\Delta\log K^*_{(Cu/Ala)}$ (c)
0	2.42±0.02	9.46±0.02	8.19±0.01	7.49±0.03	-0.70±0.03	-1.38±0.03
30	2.85±0.02	9.59±0.02	8.89±0.02	8.01±0.02	-0.88±0.03	-1.46±0.04
50	3.27±0.02	9.70±0.02	9.54±0.02	8.48±0.03	-1.06±0.04	-1.51±0.04
70	3.65±0.02	9.76±0.01	10.20±0.01	9.02±0.03	-1.18±0.03	-1.51±0.03
80	4.01±0.02	9.70±0.01	10.68±0.02	9.21±0.02	-1.47±0.03	-1.61±0.03

a) The errors given are *three times* the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. The values of the error limits for $\Delta \log K^*_{(Cu/AA)}$ were calculated according to the error propagation after Gauss [47].

b) Analogous to eq. 6 for a binary system: i.e., $\Delta \log K^*_{(Cu/Trp)} = \log K^{Cu(Trp)}_{Cu(Trp)_2} - \log K^{Cu}_{Cu(Trp)}$.

c) Analogous to eq. 7 for a binary system: i.e., $\Delta \log K^*_{(Cu/Ala)} = \log K^{Cu(Ala)}_{Cu(Ala)_2} - \log K^{Cu}_{Cu(Ala)}$.

TABLE 3. Extent of intramolecular indole stacking in $Cu(Trp)_2$ complexes [47] in dependence on the amount of dioxane added to water at 25°C and I = 0.1 M ($NaNO_3$)[a]

% (v/v) dioxane	mol fraction	ε (b)	$\Delta\log K^*_{(Cu/Trp)}$ (c)	$\Delta\log K^*_{(Cu/Trp)_{op}}$ (d)	$\Delta\Delta\log K^*$ (e)	K^*_I (e)	$\%Cu(Trp)_{2/cl}$ (e)
0	0	78.5	-0.70±0.03	-1.38±0.03	0.68±0.04	3.79±0.47	79±2
30	0.083	52.7	-0.88±0.03	-1.46±0.04	0.58±0.05	2.80±0.44	74±3
50	0.175	35.2	-1.06±0.04	-1.51±0.04	0.45±0.06	1.82±0.37	65±5
70	0.331	18.6	-1.18±0.03	-1.51±0.03	0.33±0.04	1.14±0.21	53±5
80	0.459	11.6	-1.47±0.03	-1.61±0.03	0.14±0.04	0.38±0.13	28±7

a) The errors given are *three times* the standard error of the mean value [47].
b) Dielectric constants from ref. [37].
c) See footnote 'b' of Table 2.
d) The properties of the open form are quantified by the Cu^{2+}/alaninate system: i.e., $\Delta \log K^*_{(Cu/Trp)_{op}} = \Delta \log K^*_{(Cu/Ala)} = \log K^{Cu(Ala)}_{Cu(Ala)_2} - \log K^{Cu}_{Cu(Ala)}$ (analogous to eq. 7).
e) $\Delta\Delta \log K^*$, K^*_I and $\%\ Cu(Trp)_{2/cl}$ are defined analogously to equations 9, 3 and 1, respectively, with A = B = Trp^-.

dioxane; it must again be emphasized that this observation differs from the experience with unbridged adducts [31,35] (see also Sections 2 and 3). That the indicated results for $Cu(Trp)_{2/cl}$ are no exception is seen in Fig. 9. The formation degree of the intramolecular adducts (eq. 1) decreases in the series: $Cu(Trp)_2 > Cu(Phe)_2 > Cu(valinate)_2 \gtrsim Cu(Leu)_2 \gtrsim Cu(norvalinate)_2$ [46,47].

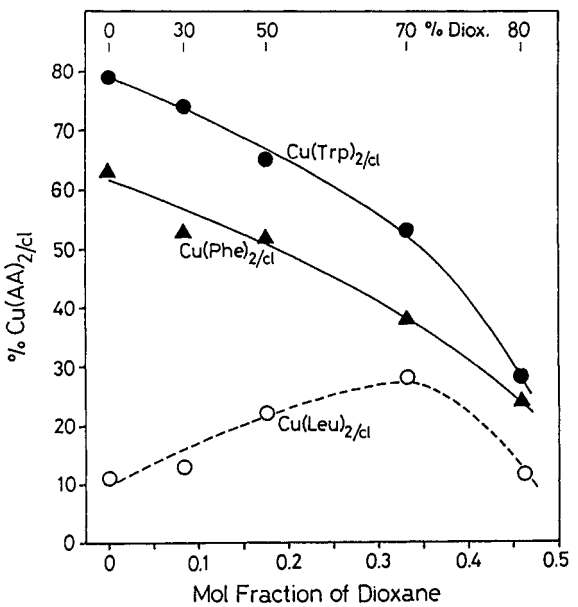


Fig. 9. Formation degree of the intramolecularly closed species (eq. 1) in the binary $Cu(Trp)_2$ (●), $Cu(Phe)_2$ (▲) and $Cu(Leu)_2$ (○) complexes in dependence on the mol fraction of dioxane in water at 25°C and I = 0.1 M ($NaNO_3$) [46,47].

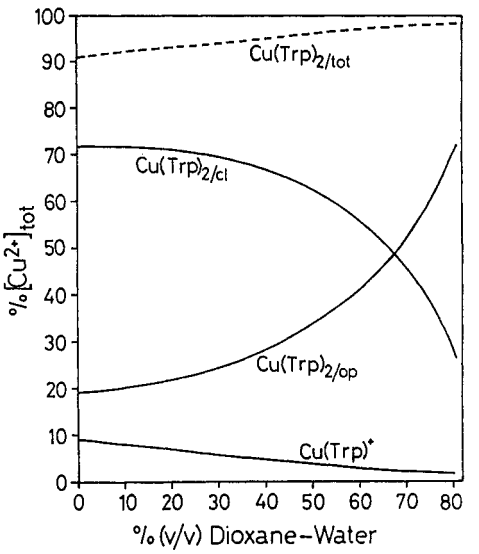


Fig. 10. Effect of the amount of dioxane added to an aqueous solution of Cu^{2+} and *L*-tryptophan on the concentration of the species present at pH 7.00 (full lines). The broken line represents the total concentration of the $Cu(Trp)_2$ complexes. The results are given as the percentage of the total Cu^{2+} concentration present (= 10^{-3} M; $[Trp]_{tot}$ = 2 x 10^{-3} M; I = 0.1 M, $NaNO_3$; 25°C) [47].

The solvent effects on the position of equilibrium 1 have already been shortly discussed in Section 3 [35,36]; the conclusions reached there for mixed ligand complexes are valid of course also for an interaction between identical ligands (A = B) in binary complexes [46,47]. However, there is one further aspect which warrants emphasis: Figure 10 shows that in the neutral pH range under the influence of dioxane the total formation degree of $Cu(Trp)_{2/tot}$ does hardly change; what is *changing* is the formation degree of the closed and open forms, i.e. of $Cu(Trp)_{2/cl}$ and $Cu(Trp)_{2/op}$ (eq. 1); in other words, the structure in which the $Cu(Trp)_2$ complex exists in solution is strongly dependent on the solvent composition.

TABLE 4. Estimations for the percentage of the closed species (eq. 1) with an intramolecular hydrophobic or aromatic-ring stacking interaction for several binary and ternary complexes of amino acids in aqueous solution at 25°C (I = 0.05-0.1 M)[a,b]

Complex	% $M(A)_{2/cl}$ or $M(A)(B)_{cl}$ for M^{2+} =			
	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}
$M(Nva)_2$	13	~2	17/ 9 ± 7	
$M(Nle)_2$	13	~4		
$M(Phe)_2$	46	38	59/63 ± 4	53
$M(Tyr)_2$	66	38	67	67
$M(Trp)_2$		48 ± 8[c]	87/79 ± 2	
M(Nva)(Abu)		~5	9	
M(Phe)(Nva)	21	~5	11	
M(Phe)(Tyr)	50	26[d]	37[e]	
M(Tyr)(Phe)		24[d]	35[e]	

a) Abu^-, α-aminobutyrate; Nle^-, norleucinate; Nva^-, norvalinate; Tyr^-, tyrosinate; see also Fig. 8.
b) The values with an error limit (3σ) are for the binary Cu^{2+} complexes with Nva^-, and Phe^- or Trp^- from references [46] and [47], respectively; all the other values for the binary complexes are the averages (in the case of several values) of the percentages listed in table VI of ref. [45]. The values for the ternary complexes are abstracted from entries No. 1-48 of table VII in ref. [13].
c) Calculated [47] with the stability constants given in ref. [48].
d,e) Note: the pairwise agreement of these data is excellent.

In amino acid complexes of metal ions with a coordination sphere different from that of Cu^{2+} is of course also a side-chain interaction possible. To emphasize this and the fact that the phenomena described here are of a general nature, the data of Table 4, which refer to aqueous solutions, have been compiled. These and all the other results given in this section are instructive because they reveal the structuring forces inherent in the side chains of tryptophan, phenylalanine and aliphatic amino acids. These forces are important in the formation of the three-dimensional structures of proteins, as well as in the recognition reactions between nucleotides or nucleic acids and amino acids or proteins.

6. CONCLUDING REMARKS

The importance of hydrophobic and stacking interactions in systems of biological origin is recognized today [23,49,50]. However, the interplay between these interactions and the coordination of metal ions is only at the brink of recognition [13,17,32,51]. The possibility of reducing the *effective* dielectric constant close to a metal ion via a nearby hydrophobic micelle (Sections 3 and 4) offers a very subtle tool for nature [35,36] to alter the polar properties of the metal ion. It should be pointed out that the energy differences (ΔG°) involved are very small, e.g. a stability increase of a complex by 0.1 log unit (see Tables 1 and 3) is connected with a formation degree of about 20% for the closed species (eq. 1) and corresponds only to -0.6 kJ/mol [4,24].

That during an enzymic reaction the *effective* dielectric constant in the active-site cavity is changing in many cases seems evident. For example, crystallographic investigations show that horse liver alcohol dehydrogenase has a deep hydrophobic pocket with the catalytic Zn^{2+} located at its bottom [52]. The adenine residue of the coenzyme, nicotinamide adenine dinucleotide (NAD), binds by hydrophobic interactions between two isoleucine side chains; in addition, ribose-hydroxyls and oxygens of the diphosphate bridge make hydrogen bonds to suitable amino acid side-chain residues of the protein [53]. Coenzyme binding to the apoenzyme is connected with a large conformational change from an 'open' to a 'closed' form [53]: there are about 40 well-ordered water molecules in the coenzyme binding cleft, and about 25 of these are displaced upon coenzyme binding and the connected conformational change. With the summarized results in mind, it is evident that here a subtle interplay between polar and hydrophobic interactions is occurring during the enzymic process.

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