Biophysical Chemistry 24 (1986) 235–247
Elsevier

BPC 01074

CHARGE REDISTRIBUTION IN PROTEINS VIA LINEAR HYDROGEN-BOND CHAINS

Alex P. KORN

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 10th October 1985
Revised manuscript received 14th April 1986
Accepted 16th April 1986

Key words: Hydrogen-bond chain; Tautomerism; Enzyme mechanism

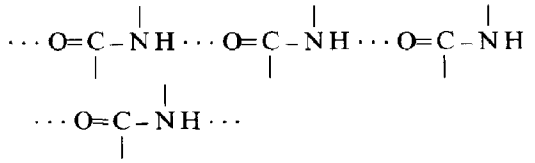
It is proposed that proteins might activate specific atomic positions within bound substrates or co-factors by means of hydrogen-bond chains. As a result of a concerted proton (tautomeric) shift in the linked residues of the hydrogen-bond chain, which includes the bound molecule, a charge separation occurs. The charge thus generated at a specific atom of the bound molecule renders it nucleophilic or electrophilic, as the case may be, and hence 'activated' towards subsequent chemical events. To test the feasibility of the theory a survey of published X-ray diffraction determined structures was performed. A search was made for hydrogen-bond chains which emanate away from bound substrates, co-factors or metal ions in order to validate the existence of such structural arrangements. Secondly, an attempt was made to incorporate the proposed proton dynamics into the proteins' mechanisms of action. Examples in which these criteria were satisfied are carboxypeptidase A, carbonic anhydrase, haemoglobin, dihydrofolate reductase, glutathione reductase and *p*-hydroxybenzoate hydroxylase.

1. Introduction

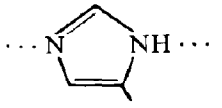
In this paper I discuss the possibility of a functional role for continuous hydrogen-bond chains in proteins. For the purpose of a generalizing definition the diagram below is presented:

$$\cdots AH \cdots AH \cdots AH \cdots AH \cdots$$

The As may represent either single heteroatoms, such as oxygen or nitrogen, or functional groups which are bivalent in their hydrogen-bonding capability. Examples of amino acids where A is a single atom (oxygen) are serine and threonine. In this case the hydrogen-bond chain would be $\cdots OH \cdots OH \cdots OH \cdots$. Examples of amino acids where A is a whole functional group are those that contain the amide group, $O{=}C{-}NH$. These are the amides of the peptide linkage and of the R groups of asparagine and glutamine. A continuous chain of hydrogen-bonded amides would have the following structure:



In addition, the imidazole ring of histidine also has the capability of bivalent hydrogen bonding:



A continuous hydrogen-bond chain need not be composed exclusively of just one type of hydrogen-bonding group; any mixture of single-atom or multi-atom functional groups as designated above can form a chain. That hydrogen-bond chains exist in proteins is trivial: α-helices and β-pleated sheets are examples that readily come to mind. That hydrogen-bond chains participate in tertiary structure is less well-known, however. Examples of these as well as of the former kind will be discussed in this paper.

Several ideas on the dynamics of a hydrogen-bond chain structure can be hypothesized. For example, quantum-mechanical calculations on linked amides [1] have indicated that, while in the ground state the system is localized, in the excited state it may become delocalized. This means that the transfer of electrons can occur from one end of the chain to the other . This has implications in photosynthesis and the visual process. However, until complete, high-resolution structures from X-ray diffraction are obtained for the proteins of these systems, we cannot know if hydrogen-bond chains exist in them, nor, a fortiori, if photo-induced semiconductivity in the hydrogen-bond chains is a real possibility. Another kind of dynamic behavior has its analogue in the phenomenon of ferroelectricity [2]. Ferroelectric materials are crystals which manifest a macroscopic polarization that can be reversed by the application of an external electric field. Most ferroelectrics are organic compounds having hydrogen bonds in their structures. The polarization of the crystal comes about from the dipole associated with the covalent hydrogen-heteroatom bond, and from the fact that the crystal structure symmetry group does not allow cancellation of the net dipoles. The reversal of the polarization comes about from the shifting of the protons from one heteroatom to the other, passing from one minimum to another of a double-welled potential energy surface.

$$AH \cdots A \rightleftarrows A \cdots HA$$

If a chain of hydrogen bonds were involved, it would result in a concerted and correlated shift of the protons in the entire hydrogen-bond chain.

$$H \cdots AH \cdots AH \cdots AH \cdots AH \cdots \rightleftarrows \cdots HA$$
$$\cdots HA \cdots HA \cdots HA \cdots H$$

If the chain were finite, the result would be a separation of charges.

$$AH \cdots AH \cdots AH \cdots AH \rightleftarrows {}^{-}A \cdots HA$$
$$+$$
$$\cdots HA \cdots H\,A\,H$$

Another mechanism for ferroelectric behaviour is a physical 180° rotation of whole groups:

$$\begin{array}{cccccccccc} A & H & \cdots & A & H & \cdots & A & H & \rightleftarrows & H\,A \cdots H\,A \cdots H\,A \\ | & & & | & & & | & & & | \quad | \quad | \end{array}$$

For either mechanism the ferroelectric shift is characterized by concertedness and co-operativity when undergoing transition from one state to another. For a biological macromolecule with hydrogen-bond chains, the analogue of an externally applied electric field would be some chemical agent – a ligand – that, when bound to one end of the chain, causes a local pH disturbance, due to its carrying a charged group, breaks the chain and reverses the direction of hydrogen bonding. This means that the event of ligand binding might be felt at a large distance from the site of the primary event, and that the residue at the other end of the chain will be switched by a signal whose transmittance will not have dissipated with distance. It is also feasible that transmittance of perturbation can occur by a physical shift of all groups involved in the hydrogen-bond chain, provided that other interactions permit the conformational re-adjustment. Thus, a ligand, whose binding physically distorts a terminal residue, may cause the subsequent re-adjustment in the physical position of the residue which is hydrogen bonded to the terminal one and, in turn, affect the second, third and so on in order to maintain the integrity of the entire hydrogen-bond chain.

To test the feasibility of these ideas, a survey of X-ray diffraction determined structures of proteins was performed to scan for hydrogen-bond chains. The survey was done in large part using computer molecular graphics and other published reports. The study was restricted to proteins whose interactions with ligands were reasonably well characterized structurally. It consisted of searching the residues of the binding site for hydrogen-bonded connectivities that emanate away from the ligand site and which may even arrive at a functional group that may transmit some influence back to the ligand through the hydrogen bonds. The molecular graphics software, IMDAD, was written by Dr. Michael Levitt for the VAX 780. The criterion for a hydrogen bond was a distance between heteroatoms of not more than 3.4 Å. The co-ordinate data were obtained from the Brookhaven Protein Data File. Surveys with IMDAD were done with data updated to 1983 except for carboxypeptidase A which was from the 1984 file. Hardcopy stereodiagrams were drawn by the

PLUTO program written by Sam Motherwell which runs on the IBM 3081. The rationale for such an approach is that if concerted proton transfer along hydrogen-bond chains can be successfully incorporated into the mechanism of action of an enzyme then the feasibility of the theory can be established.

2. Examples

2.1. Carboxypeptidase A

Carboxypeptidase A (5CPA) is a hydrolytic enzyme that catalyzes the cleavage of carboxy-terminal peptides and esters. A Zn^{2+} which is bound at the catalytic site is necessary for the hydrolysis reaction, but the precise detailed mechanism by which it plays its role is not known [3]. Fig. 1 is a stereodiagram showing a selection of groups in the immediate environment of the Zn^{2+}. It shows a hydrogen-bonded connectivity from His 196, which ligates the zinc, to a buried water molecule (568), to a second buried water molecule (564) and finally to the carboxylate group of Asp 65. (The hydrogen-bonded pair of waters was noted by Rees et al. [4] but was not commented on in relation to any possible functional significance.) Fig. 2 presents the system in schematic form and in two 'ferroelectric' states. The tautomeric isomerization of imidazole suggests that a negative charge can be brought to the zinc and, in turn, to water 571 (not shown) which is ligated to the zinc. The negative charge endows this water molecule

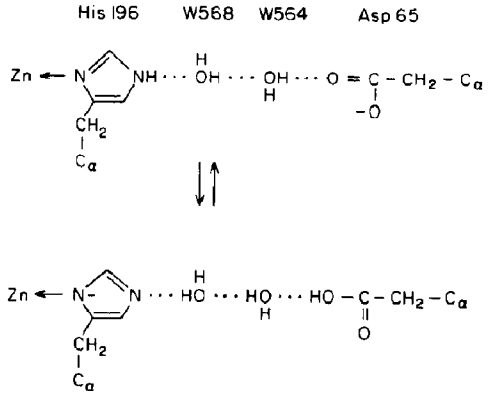


Fig. 2. Proposed proton shift in the hydrogen-bond chain in carboxypeptidase A. The schematic diagram is based on the geometrically accurate sterodiagram in fig. 1.

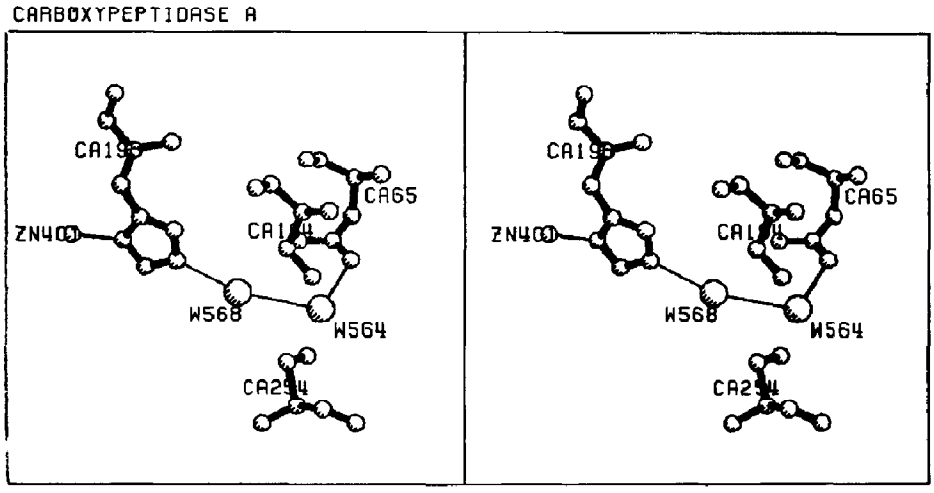


Fig. 1. Stereodiagram showing hydrogen-bonded connectivity in the catalytic site of carboxypeptidase A. Thin continuous lines indicate the ligation between the Zn^{2+} ion and the imidazole of His 196, and the hydrogen bonds between imidazole of His 196 and water 568, water 568 and water 564, and water 564 and carboxylate (O1) of Asp 65. Other residues included in the diagram are Ser 194 and Ser 254 which might also be hydrogen bonded to W564.

with enhanced nucleophilicity. It may then proceed to act as a specific base attacking the scissile bond of the peptide or ester substrate. The possibility that zinc by itself may enhance the nucleophilicity of a water molecule bound to it has been postulated before (p. 410 of ref. 3) but until now it has not been suggested that its nucleophilicity could be enhanced by the distant negative charge of Asp 65 via a hydrogen-bond chain.

2.2. Carbonic anhydrase

Carbonic anhydrase is another zinc-containing enzyme that catalyzes the conversion of soluble CO_2 to bicarbonate and the hydrolysis of some esters. The X-ray determined structure of human erythrocytic carbonic anhydrase B [5] demonstrates a remarkable hydrogen-bonded network involving the Zn^{2+}. Fig. 3 shows part of it in schematic form. (The original stereodiagram can be found in fig. 3 of ref. 5.) On the one side of zinc the ligated His 119 is hydrogen-bonded to Glu 117. On the other side, a solvent water molecule bound to the zinc is hydrogen-bonded to Thr 199 which in turn is hydrogen-bonded to buried Glu 106. Now, it is proposed that the negative charge of Glu 106 (as well as Glu 117) may be felt on the zinc via proton shifts along the hydrogen-bond chain. It has already been suggested [3] that zinc enhances the nucleophilicity of a bound water molecule which subsequently attacks CO_2. In view

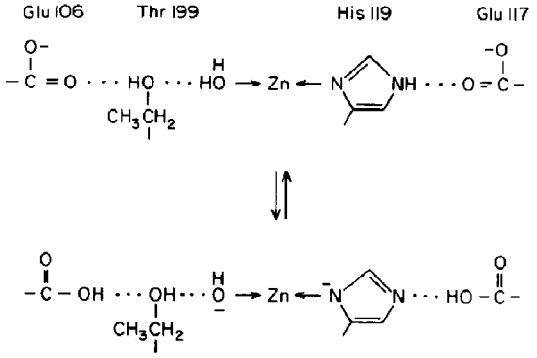


Fig. 3. Schematic diagram of hydrogen-bond chain in carbonic anhydrase and the postulated proton shift that brings a partial negative charge from Glu 106 to zinc.

of the indirect linkage to Glu 117 via hydrogen bonding to His 119 it may be suggested that zinc's nucleophilicity may be enhanced by the partial negative charge of Glu 117. In addition, the hydrogen-bond chain to Glu 106 suggests a proton shift that might already coax the bound water molecule to split into H^+ and OH^-. The product, OH^-, may then act as a specific base in the attack on the substrate. Thus, from both directions, from the zinc's enhanced nucleophilicity and the hydrogen-bond chain to a distant negatively charged group, we witness the promotion of the production of OH^- from the zinc-bound water.

This brief outline has been suggested by others and is discussed in much detail by Kannan et al. [6] and Allen [7]. The latter author also suggested a role for an additional hydrogen-bond chain to serve as a proton-relay system in a step subsequent to bicarbonate formation. The feasibility of the hydrogen-bond chain of Glu 106, Thr 199 and bound water has also been demonstrated by the criteria of quantum-mechanical calculations [8].

2.3. Haemoglobin

Equine aquomethaemoglobin [9] was found to have a hydrogen-bond chain which creates a linkage from the iron atom of the haem group in the α_1-subunit to a salt bridge between the α_1-subunit and the β_2-subunit. Fig. 4 is a stereodiagram of this hydrogen-bond chain and fig. 5 shows it again in schematic form. The imidazole (His F8) which is ligated to the iron atom is hydrogen-bonded to the carbonyl of Leu F4. This same carbonyl group is also hydrogen-bonded to the peptidyl NH of His F8. The carbonyl (of Leu F7) of this peptide group is in turn hydrogen-bonded to the peptide NH of Leu FG3, and the carbonyl of this peptide (Lys FG2) is hydrogen-bonded to the guanidino group of Arg FG4. This positively charged group is in a salt bridge with the carboxylate of Asp CD2 which belongs to the neighbouring β_2-subunit. From the iron atom to this carboxylate the distance spanned is almost 18 Å. The hydrogen-bond chain forms an almost straight line directed towards the haem of the β_2-subunit. These hydrogen bonds were found by the authors Ladner et al. [9] but were indicated in separate figures (their

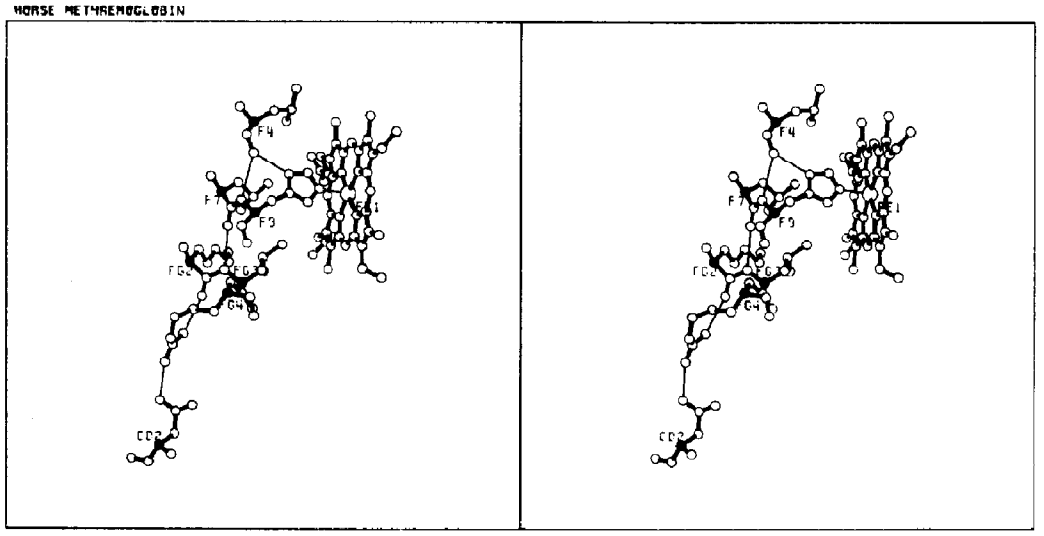


Fig. 4. Stereodiagram of the hydrogen-bond chain in haemoglobin that links iron of the haem group to a salt bridge between a residue of the α_1-subunit to a residue of the β_2-subunit.

figs. 9, 10, 12 and 19); thus, the continuity of the chain was apparently not noticed.

It has been suggested, with support from ab initio molecular orbital calculations [10], that ligand binding (and oxidation) to iron will be felt on the hydrogen bond between the imidazole and the peptide carbonyl of Leu F4. The ligation event drains negative charge out of the imidazole ring and affects the hydrogen bond by increasing its strength. In fact, these authors observed from the

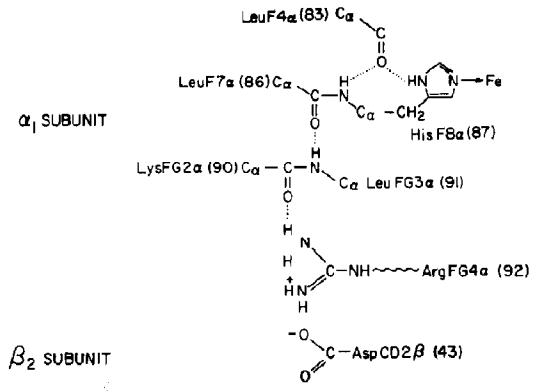


Fig. 5. Schematic diagram of the hydrogen-bond chain in haemoglobin.

X-ray structure co-ordinates that the protein manifests a hydrogen-bond geometry here which is more favourable in the oxidized form than in the reduced form. They suggested further that the coupling can work in the reverse direction; i.e., that a change in the hydrogen-bond geometry may alter the affinity of the iron for ligand. The R (high-affinity) state then is a conformation which is characterized by the favourable hydrogen-bond geometry which causes the iron to have a high affinity for oxygen. The question that remained unanswered, though, was how the subunits communicated the transition among each other. The chain of hydrogen bonds suggests a partial explanation. Subsequent to the first ligation step, the shift in geometry of the imidazole Leu F4 carbonyl hydrogen bond will affect sequentially the other hydrogen bonds in the chain until the salt bridge, Arg FG4 to Asp CD2, between subunits α_1 and β_2. That this salt bridge is important in inter-subunit communication is demonstrated by the fact that human haemoglobin Chesapeake has a Hill constant of 1.3 [11] indicating very low co-operativity. This haemoglobin has Arg FG4 replaced with Leu, thus making the salt bridge impossible. The hydrogen-bond chain in haemoglobin does

not continue all the way to the iron of the β-subunit from the iron of the α-subunit, however. Thus, the mechanism of inter-subunit co-operativity cannot be exclusively due to this hydrogen-bond chain; other effects must be involved as well.

The β-subunit of equine methaemoglobin was examined as well and was found to contain an analogous hydrogen-bond chain up to Lys FG2. It ends there, however, and does not make further hydrogen-bonded or salt-bridge contacts. A control was performed on myoglobin, which is monomeric and does not bind oxygen co-operatively. Of six X-ray diffraction-solved structures in the (1983) Brookhaven Protein Data Bank, three displayed the hydrogen-bond chain. The three that did not had the relevant groups farther apart than what was acceptable for proper hydrogen bonds. Thus, it cannot be said that the control was decisively in favour or decisively against the proposed role of hydrogen-bond chains in haemoglobin. It may be worth mentioning that other approaches (ref. 12 and other papers cited within) have assumed that it is the tertiary and quaternary structural changes that transmit the information of ligation from subunit to subunit and that bring about the high-affinity state. How these conformational changes are supposed to alter oxygen affinity is not made totally clear, however. A mechanism such as one involving hydrogen-bond chains, on the other hand, has as its primary event a shift of protons and electron densities that become felt on the iron atom. It therefore has the advantages of rapidity and directness, and, on a philosophical level, it may possess greater explanatory power. In addition, information transmission by steric-induced conformational change has been shown to be limited in that the effect dissipates with distance [13]. Transmission along hydrogen-bond chains, on the other hand, would tend not to dissipate so readily by virtue of their co-operativity and concertedness. If the hydrogen-bond chain participation is true, what then would be the role of the (proven) tertiary and quaternary conformational changes which involve whole residues, domains and subunits? These conformational changes may be a secondary event, i.e., occurring chronologically after the true T ⇄ R transition – and might serve only to stabilize the resultant high-affinity state.

2.4. Dihydrofolate reductase

Dihydrofolate reductase catalyzes the redox reaction in which the substrate 7,8-dihydrofolate is reduced to 5,6,7,8-tetrahydrofolate and is accompanied by the oxidation of NADPH to $NADP^+$. Part of the mechanism is believed to involve a protonation of dihydrofolate, where the proton originates from solvent water. Next, a hydride transfer to the protonated pteridine occurs. The origin of the H^- is probably the nicotinamide ring of $NADPH_2$. The loss of the H^- results then in the oxidation of NADPH to $NADP^+$ [14]. The detailed sequence of events is uncertain largely because no ternary complex comprising enzyme, cofactor and substrate has been crystallized; information has been obtained from complexes with inhibitors such as methotrexate.

An examination of the X-ray determined structure of *Lactobacillus caseii* dihydrofolate reductase (3 DER) revealed a hydrogen-bond chain which is hydrogen-bonded to the carboxamide group of

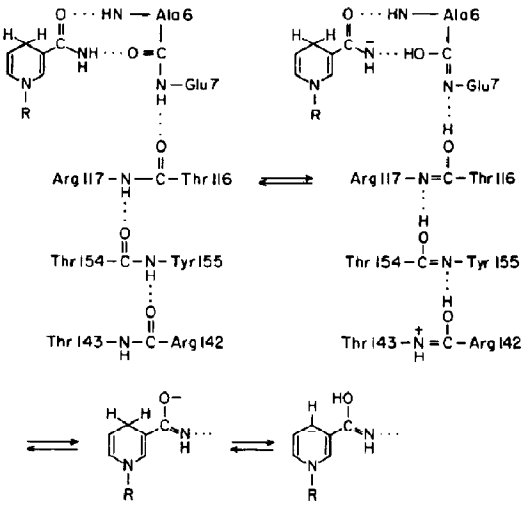


Fig. 6. Schematic representation of the hydrogen-bond chain in dihydrofolate reductase and postulated tautomeric and resonance structures associated with it. These transients can bring a partial negative charge to the region of carbon 4's hydrogen thereby facilitating its removal as an H^-. Subsequent to hydride removal, the proton shift in the hydrogen-bond chain relaxes. In the relaxed state a positive charge is deposited in the nicotinamide ring and the oxidized state is established.

nicotinamide (see fig 6). The hydrogen-bond chain from Glu 7 to Arg 142 is part of a large hydrogen-bond network of a β-pleated sheet located in this corner of the molecule. There is another hydrogen bond involving the NH of Ala 6 and the carbonyl of the carboxamide group. What would happen if a proton shift were to occur? Fig. 6 shows the chain in the alternate resonance forms and demonstrates that a dipole can reside in the chain. It places a partial positive charge on the carbonyl of Arg 142 and a partial negative charge on the NH_2 of the nicotinamide. Further resonance structures suggest themselves which place partial negative charges on the oxygen, the C4 of the ring and the nitrogen of peptide Ala 6 (not shown). These transient structures suggest a mechanism for the creation of an H^-. With C4's hydrogen loosened it can be captured by C7 of the pteridine ring of dihydrofolate. According to the model of Filman et al. (see fig. 4 of ref. 14) these carbons are sufficiently close to make this transfer feasible. With a positive charge (which will rearrange and re-distribute to N1) now residing in the ring, NADPH has undergone oxidation to $NADP^+$.

At this point one might ask if the contribution of these resonance structures be sufficiently important to promote hydride transfer. It has been noted that, with regard to the hydrogen-bond chain of the α-helix, the helix dipole is greater by about 14% than what one would expect due to superposition of independent dipoles alone. This is because of polarization of linearly arranged dipoles [15]. Even without considering this enhancement, the authors suggested that it is "likely that in some enzymes the field of the helix plays a part during catalysis". The thermodynamic contribution was estimated to be about 10 kcal/mol, which is comparable to activation energies which reach about 20 kcal/mol. There is a priori no reason that the electric dipole associated with any hydrogen-bond chain should not also enjoy such enhancement. Therefore, it is suggested that the strength of the dipole associated with the hydrogen-bond chain in dihydrofolate reductase may be sufficiently strong to promote partial negative charges on the oxygen of the carboxamide, nitrogen of peptide Ala 6 and C4 of the nicotinamide ring. The entire mechanism, of course, must be understood by considering the enzyme-induced activation on the pteridine ring as well.

2.5. *Glutathione reductase*

Glutathione reductase catalyzes the hydrogenation of glutathione disulphide dimers at the expense of oxidation of NADPH to $NADP^+$. The cofactor, flavin adenine dinucleotide (FAD), mediates the transfer of reduction equivalents from nicotinamide to the enzyme's disulphide bond be-

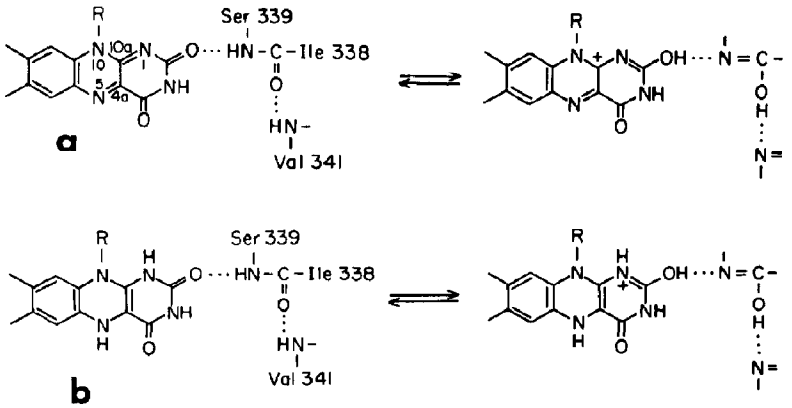


Fig. 7. (a) Hydrogen-bonded connectivity between the oxidized form of the flavin ring and hydrogen bond chain of α-helix 338–359 in glutathione reductase. The entire hydrogen-bond chain of the helix is not shown. The tautomeric shift induces a positive charge at position C10a, thereby promoting it as a site for possible nucleophilic attack. In the case of the reduced form (b) of the flavin ring, the tautomeric shift places a positive charge on N1 thereby increasing its acidity.

tween Cys 58 and Cys 63. The passage of reduction equivalents then proceeds on to the disulphide bond of the glutathione dimer. The enzyme has been co-crystallized with co-factor and substrate and at different stages of the process by Pai and Schultz [16]. The structure (2GRS) was scanned for hydrogen bonds using the molecular graphics display. The survey revealed a hydrogen bond between the carbonyl oxygen (C2) on the flavin ring and NH of the peptide of Ser 339 (see fig. 7a). Now, this peptide is one of a seven-membered chain of hydrogen bonds which is part of α-helix 338–359. If we bring about a ferroelectric-like shift of protons along the hydrogen-bond chain we promote a partial positive charge on position C10a of the oxidized form of the flavin ring. Therefore, this position might be a site of nucleophilic attack. Furthermore, the intermediate adduct resulting from such nucleophilic attack would be stabilized by the hydrogen-bond chain by absorbing the negative charge into it. Quantum-mechanical calculations performed by Pullman and Pullman [17] indicate that the intrinsic electron charge on C10a (0.883) is less than that of C4a (0.979). This suggests that C10a is intrinsically a site of nucleophilic attack. However, the more sophisticated calculations of Sun and Song [18], which take into consideration other quantum-mechanically derived properties, show greater susceptibility of C4a to nucleophilic attack. Taking into account the possible influence of the hydrogen-bonded chain, however, it may be that electron densities are altered sufficiently to make C10a decisively more reactive than C4a. Calculations on FAD [17,18] predicted that nitrogens N1 and N5 differ widely in their basicities with N1 being the more basic. Thus, if the first step of reduction consists of protonation, it would more readily occur at N1. Calculated results [17,18] on protonated FAD ($FADH^+$), incidentally, show C10a to be more reactive to nucleophiles than C4a. Thus, if protonation is the first step of reduction, nucleophilic adduct formation would proceed via C10a.

Now, in the reduced form ($FADH_2$) of the flavin ring the greater electron-withdrawing power of position C10a over C4a is maintained; in fact, the differential is widened further compared to $FADH^+$. Thus, nucleophilic attack at C10a is intrinsically more favourable. The resonance structures promoted by the hydrogen-bond chain do not, however, affect these carbon positions. On the other hand, the relative acidities of N1 and N5 in $FADH_2$, which are almost identical [17], are affected by hydrogen-bond chain-promoted resonance structures (fig. 7b): the acidity of N1 can be enhanced over that of N5. In summary, the influence of the hydrogen-bond chain in the flavin-reduction step would be to promote the nucleophilic attack on C10a, and, in the flavin-oxidation step, to increase the leaving power of the proton on N1. Fig. 8 presents a suggested mechanism for the reduction of FAD by NADPH which includes the postulated participation of the hydrogen-bond chain in promoting the reaction and stabilizing an intermediate.

Before closing this section it must be remarked that the current literature favours C4a for both nucleophilic attacks: that of the oxygen of the carboxamide of NADPH in the flavin-reduction step and that of the thiolate of Cys 63 in the subsequent oxidation step (see, for instance, ref. 19). The arguments cited in favour of the mechanism were based on spectral and kinetic experiments on solvent-dissolved model compounds [20–22]. The evidence, therefore, is essentially inferential in nature. If it is indeed true that in these experiments C4a is the site of nucleophilic attack in free solution, then it can be attributed to the steric blocking of C10a by bulky substituents on position N10. The solid angle of unhindered approach of reagents to C10a is indeed reduced compared to that of C4a. However, the X-ray crystallographic determined geometry of the reacting groups reveals the following. The carboxamide group of the nicotinamide ring is rotated by about 20° with respect to the plane of the ring thereby tilting the carbonyl's oxygen (the nucleophile) towards the flavin ring. Now, this geometry (see figs. 1 and 4 in ref. 16) favours a (transient) covalent bond with C10a rather than with C4a. The angle made by carbonyl carbon, carbonyl oxygen and carbon C4a is not favourable for a covalent intermediate with C4a. Now, as for the oxidation step, the X-ray data also show that the sulphur atom of Cys 63 is in close contact with

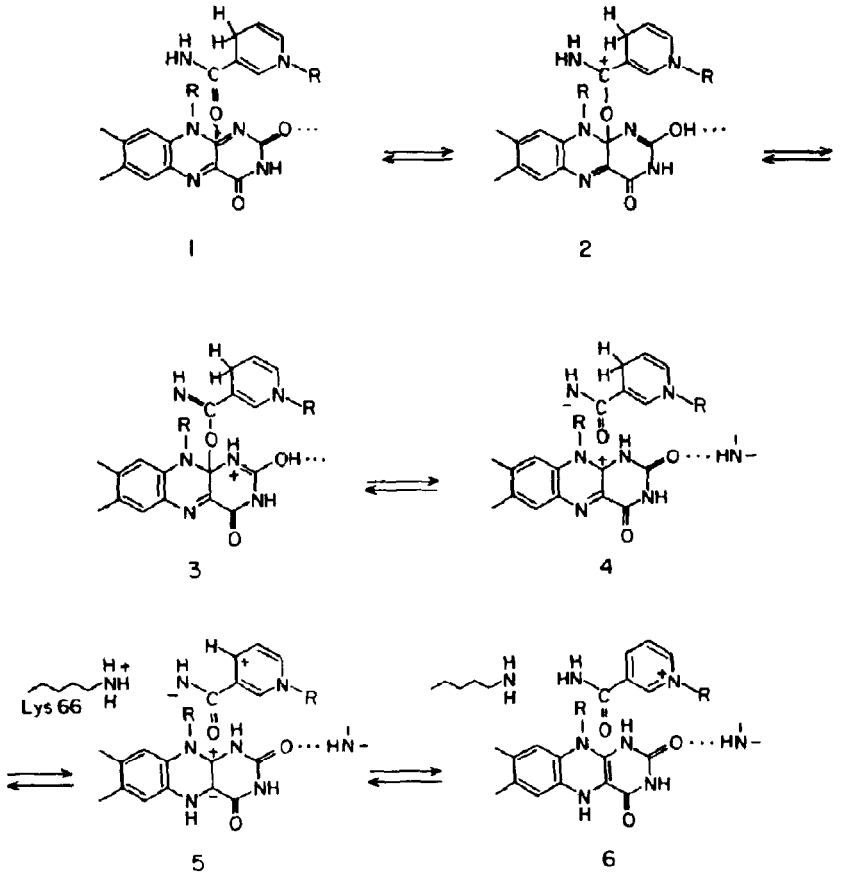


Fig. 8. Various stages in the reduction of flavin by NADPH showing how the hydrogen-bond chain might increase stability of the intermediate covalent adduct. The seven-membered hydrogen bond chain belonging to α-helix 338–359 (not shown) is linked to carbonyl O2. Stage 1 indicates nucleophilic attack of the carbonyl oxygen of nicotinamide at carbon 10a of the flavin ring. (For exact geometry see fig. 1 of ref. 16.) At stage 2 the negative charge that would ordinarily have been created at oxygen 2a is absorbed into the hydrogen-bond chain. The charge finds itself ultimately at the carbonyl of Tyr 356. Several stages have been compressed at stage 3. The source of the proton at N1 may be the NH_2 of carboxamide which contributes it via the carboxamide oxygen. (Alternatively, protonation of N1 might have preceded stage 1, in which case the proton source would be solvent water.) In stage 4 the polarization induced by the hydrogen-bond chain is relaxed and the transient covalent bond between nicotinamide and flavin is broken. The stability of one of the hydrogens on C4 of the nicotinamide is diminished by the proximity of NH^-. This promotes the leaving of an H^- from C4 and arrival at the nearby N5 of the flavin ring. This process neutralizes the flavin ring and completes its reduction. Lys 66 is close enough to be able to donate a proton to NH^- of nicotinamide, thereby completing its oxidation at stage 6.

the flavin ring (its perpendicular distance is 3.4 Å from the plane of the ring) and lies halfway between C4a and C10a; and, that it approaches closer to the ring by 0.1 Å after the release of $NADP^+$ from the enzyme. Clearly, nothing prevents approach to either carbon in spite of the steric hindrance of the R group of D-ribitol on N10. Steric hindrance is irrelevant here because it operates mainly within the plane of the flavin ring whereas the reacting agents are above and below the ring. The choice that is made is only on the basis of relative charge densities and reactivities as discussed above. The disadvantage of free solution studies with model compounds is that they do not

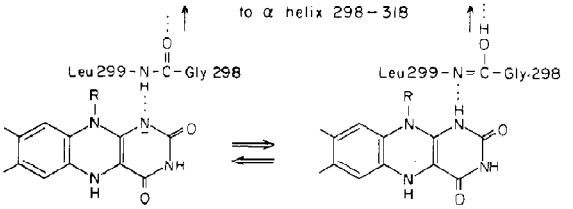


Fig. 9. Proposed mechanism for the stabilization of the negative charge on N1 of reduced flavin in *p*-hydroxybenzoate hydroxylase. The negative charge is created at an early stage in the mechanism [26]. The hydrogen-bond chain, part of helix 298–318, absorbs the charge into the chain by means of a concerted tautomeric shift and thereby stabilizes the charged flavin intermediate. The most distant possible site of the negative charge is the carbonyl oxygen of residue 316.

take into account the precise and specific geometry forced on substrates by an enzyme's catalytic site structure.

2.6. p-Hydroxybenzoate hydroxylase

The X-ray structure of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* [23] indicates a hydrogen bond connecting N1 of the flavin co-factor to the NH of the peptide of Leu 299. Now, Leu 299 is part of α-helix 298–318. Thus, we have a linear, continuous hydrogen-bond chain composed of N1 of flavin, peptide 298–299, peptide 301–302, 304–305, 307–308, 310–311, 313–314 and 316–317. It has been suggested [24] based on spectral evidence that the reduced flavin's N1 acquires a negative charge during catalysis. (For the detailed proposed mechanism see ref. 24.) The hydrogen-bond chain may serve to stabilize the charge on N1 by absorbing it into the chain during the concerted tautomeric shift (see fig. 9). Although this places a formal negative charge on the last peptide of the chain, the charge may in reality be distributed diffusely along the chain. In this and in previous examples the chain may be thought of as acting as a buffer tank for charge thereby stabilizing a charged intermediate.

3. Discussion

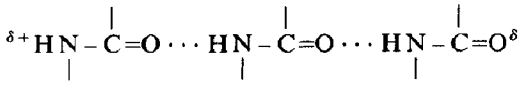
We often find in the scientific literature explanations for changes in enzyme activity or receptor response in terms of conformational change. Usually, it is not stated by what detailed mechanism this proposed change in conformation is supposed to affect the protein's activity. There is, in fact, a severe scarcity of examples where an actual, observed conformational change has supplied a meaningful and complete account of mechanism of action. In spite of this, many workers will resort to 'conformational change' as if this phrase in itself has sufficient explanatory power. Instead, it seems to this author that the complacent use of this catch phrase actually blocks the way to a deeper understanding.

In this work, the dynamical aspects of a structural feature – hydrogen-bond chains – has been considered. The expected dynamics of a hydrogen-bond chain involve concerted proton (and concomitant electron) shifts along the length of the chain, rotation of the hydrogen-bonded groups and shifts of electrons. The latter, though not classifiable as a conformational change, was not investigated because quantum-mechanical calculations predict no flow of electrons in a continuous chain of hydrogen bonds when in the ground state, and because the structure of photosynthetic and visual proteins that may become excited during the course of their function have not been solved with sufficient accuracy. To demonstrate concerted rotation of hydrogen-bonded groups would require X-ray structures of an enzyme in two such states of activity. This also is unavailable. Thus, we were left to test the proton shift hypothesis which is, strictly speaking, a conformation change though on a modest scale.

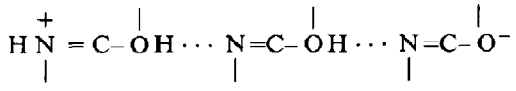
How feasible is the proposal? A proton shift along a linear chain of hydrogen-bonded groups is no more exotic than a series of tautomeric isomerizations along the length of the chain. Because of their mutual linkages the isomerizations would be concerted and co-operative. For a hydroxyl group both 'ferroelectric' states are thermodynamically equivalent. Scheiner [25] has performed quantum-mechanical calculation for hydroxyl chains where the hydroxyls may include bound water molecules as well as the protein's serines and threonines. While his motive was to explain proton transport across membranes his feasibility study is perfectly applicable to our purpose. The

thermodynamics of keto-enol isomerization in a single amide group indicates that the keto form is energetically preferred. What is being discussed here, however, is a keto-enol isomerization where a chain of many individual amide groups are involved. The proton of the NH is not the proton that transfers to the carbonyl of the same amide group. Rather, it is a neighbouring amide which is hydrogen-bonded to it that contributes the proton. The distance of travel being thereby so much less (< 1 Å), the rate of the keto-enol tautomerization in a hydrogen-bond chain would be expected to be faster than in an isolated amide group. As for the equilibrium thermodynamics, keto-enol energetics may be influenced by the neighbouring hydrogen-bonded members. Calculations similar to those of Scheiner [25] on linked hydroxyls and experimental studies on model systems would have to be done to see if the equilibrium shifts to the enol form.

Related to this issue may be the fact that the electric dipole of the amide groups in a hydrogen-bond chain either in α-helices or β-pleated sheets is enhanced relative to the monomeric amide [26]. In both approaches a partial positive charge is created at the N-terminus of the chain and a partial negative charge at the C-terminus. In the dipole field approach the following description holds:



In the tautomer model the chain is in equilibrium with:



The two approaches may be merely different descriptions of the same phenomenon. The advantage of the tautomeric shift approach is that specific sites of substrates and co-factors are affected. The electric dipole field approach, on the other hand, is argued [26] to affect a diffuse region located within the environs of the dipole, and any active-site residue located there will fall under its influence. Of the ten examples of such active-site α-helices listed by Hol [26], two (in glutathione reductase and hydroxybenzoate hydroxylase) have been discussed in this work in terms of the tautomeric shift model. If, indeed, the two models are different approaches to describe the same phenomenon, then, just as the electric dipole field of an α-helix is enhanced, by virtue of the amides being hydrogen-bonded to each other, the amides' keto-enol isomerization may be shifted more toward the enol form. Experimental tests of the hypothesis may be made by checking for kinetic isotope effects: proton shift dynamics will surely be altered strongly by replacing hydrogen with deuterium. Another test might comprise checking of pK_a values of essential catalytic groups. If a titratable active-site residue is linked to a hydrogen-bond chain then there may be an altering of its pK_a due to the charge generated in the enol state. An example which may support this claim is triose-phosphate isomerase [26] whose catalytic His 95 is hydrogen-bonded to an α-helix. Its expected pK_a is not observed in the dependence of catalytic rate on pH, presumably because the hydrogen-bond chain and its associated charge-creating keto-enol shift has lowered the imidazole's effective pK_a.

Proton dynamics in hydrogen-bond chains have been speculated upon by others for various purposes. From our discussion on carbonic anhydrase we have seen that previous authors have considered the feasibility from the point of view of quantum-mechanical calculations and incorporated the postulated proton dynamics into a mechanism of catalysis. It is noteworthy that the approach used in this work – surveying X-ray structures – succeeded in discovering the same example and proposing the same mechanism. This should strengthen our reliance on this approach in its uncovering of other examples. The transport of protons across membranes by hydrogen-bond chains in membrane proteins was suggested by Scheiner [25] who showed the extent of its feasibility by quantum-mechanical calculations. More recently (in fact, subsequent to the submission of an earlier draft of this manuscript), Tüchsen and Woodward [27] published a proposal that charge delocalization within chains of hydrogen-bonded amides might catalyze hydrogen-tritium exchange in buried NH groups and thus explain certain

anomalies in observed exchange rates. In summary, from several approaches proton shifts in hydrogen-bond chains have been suggested for various purposes: enzyme catalysis, co-operative oxygen binding in haemoglobin, hydrogen-tritium exchange, and proton transport across membranes. Can we speculate on even more possibilities? We can imagine that subunit association in multimeric systems may be regulated from the external environment via hydrogen-bond chains, communication of external disturbances, such as a change in pH or binding of a charged ligand, via proton shifts to internal, key, ionizable groups that are situated at the inter-subunit contact surface. The subsequent changes in their effective pK_a values also changes the strength of their interaction with groups on the neighbouring subunit(s). The result may be the dissociation of the subunits. If the scheme sounds familiar it is because it presents a mechanism of allostery where the conformational change is merely a proton shift in a hydrogen-bond chain.

The question now is the generality of the phenomenon. We have seen how other workers have applied hydrogen-bond chains to explain a variety of phenomena. In the present work, the relatively small number of examples should not be used to argue against its generalization. The number is small because of the stringency of the criterion: we have required that the hydrogen-bond chain be linked to a bound molecule or metal ion. The number of X-ray structures of enzymes co-crystallized with their substrates or co-factors is small and so must also be the number of potential examples that can satisfy the criteria. On the other hand, one should consider that essentially every protein has a network of hydrogen-bond chains sequestered within the structure (as α-helices, β-pleated sheets, at least) and that every chain has the possible potential of delocalizing charge, transporting protons and of being a 'communication channel' connecting distant sites. In the light of these statements, the question of the generalization of the phenomenon should require serious consideration.

4. Summary

It was shown that, subsequent to proton shift along a hydrogen-bond chain, (a) charges form at either end of the chain and (b) new resonance structures can be drawn within substrates whereby specific atomic positions in the substrates can be activated. The successful incorporation of this proposal into the mechanisms of action of carboxypeptidase A, carbonic anhydrase, glutathione reductase, dihydrofolate reductase and *p*-hydroxybenzoate hydroxylase argues well in its favour. An important aspect of the proposed role of hydrogen-bond chains is that they activate specific sites at a distance: the residues that take part in the chain extend far away from the catalytic site. Incidentally, this might serve to explain why enzymes are so much larger than what seems to be required based solely on the catalytic site residues.

Future studies will have to consist of documenting more examples. What is also hoped for is the finding of an example of an allosteric effector, or some other kind of activity-modifying factor, bound to a protein and linked via a continuous chain of hydrogen bonds to a distant substrate found at the opposite end of the protein. As it was true in 1941 it is still true now that "only the accumulation of a great mass of data will answer these questions" [28].

Acknowledgements

I would like to thank Dr. Michael Levitt for making available to me his IMDAD software and the molecular graphics system. I am indebted to Dr. Edward Trifonov for encouraging me to develop the initial idea. I thank Drs. M. Levitt, J. Sussman and M. Shoham for their constructive suggestions.

References

1 B. Pullman and A. Pullman, Quantum biochemistry (Interscience, New York, 1963) p. 308.
2 W.C. Hamilton and J.A. Ibers, Hydrogen bonding in solids (Benjamin, New York, 1968) ch. 7.

3 B.C. Vallee and A. Galdes, Adv. Enzymol. 56 (1984) 408.
4 D.C. Rees, M. Lewis and W.N. Lipscomb, J. Mol. Biol. 168 (1983) 382.
5 K.K. Kannan, B. Nostrand, K. Fridborg, S. Lövgren, A. Ohlsson and M. Petef, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 51.
6 K.K. Kannan, M. Petef, K. Fridborg, H. Cid-Dresdner and S. Lövgren, FEBS Lett. 73 (1976) 115.
7 L.C. Allen, Ann. N.Y. Acad. Sci. 367 (1981) 388.
8 A. Pullman, Ann. N.Y. Acad. Sci. 367 (1981) 340.
9 R.C. Ladner, E.J. Heidner and M.F. Perutz, J. Mol. Biol. 114 (1977) 385.
10 J.S. Valentine, R.P. Sheridan, L.C. Allen and P.C. Kahn, Proc. Natl. Acad. Sci. U.S.A. 76 (1969) 1009.
11 H. Gibson and R.L. Nagel, J. Biol. Chem. 249 (1974) 7255.
12 B.R. Gelin, A.W. Lee and M. Karplus, J. Mol. Biol. 171 (1983) 489.
13 C. Chothia, A.M. Lesk, G.G. Dodson and D.C. Hodgkin, Nature 302 (1983) 500.
14 D.J. Filman, J.T. Bolin, D.A. Matthews and J. Kraut, J. Biol. Chem. 257 (1982) 13663.
15 W.G.J. Hol, P.T. van Duijen and H.J.C. Berendsen, Nature 273 (1978) 443.
16 E.F. Pai and G.E. Schultz, J. Biol. Chem. 258 (1983) 1752.
17 B. Pullman and A. Pullman, Quantum biochemistry (Interscience, New York, 1963) p. 542.
18 M. Sun and P.S. Song, Biochemistry 12 (1973) 4663.
19 G.A. Hamilton, in: Progress in bioorganic chemistry, vol. 1, eds. E.T. Kaiser and F.J. Kezdy (Wiley-Interscience, New York, 1971) p. 108.
20 C. Thorpe and C.H. Williams, jr, J. Biol. Chem. 251 (1981) 7726.
21 I. Yokoe and T.C. Bruice, J. Am. Chem. Soc. 97 (1975) 450.
22 E.L. Loechler and T.C. Hollocher, J. Am. Chem. Soc. 102 (1980) 7312.
23 W.J. Weijer, J. Hofsteenge, J.J. Beintema, R.K. Wierenga and J. Drenth, Eur. J. Biochem. 133 (1983) 109.
24 B. Entsch, D.P. Ballou and V. Massay, J. Biol. Chem. 251 (1976) 2550.
25 S. Scheiner, Ann. N.Y. Acad. Sci. 367 (1981) 493.
26 W.G.J. Hol, Prog. Biophys. Mol. Biol. 45 (1985) 149.
27 E. Tüchsen and C. Woodward, J. Mol. Biol. 185 (1985) 421.
28 A. Szent-Gyorgi, Science 93 (1941) 609.