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Temperature adaptation of lactate dehydrogenase

Structural, functional and genetic aspects

H. Zuber

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

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Comparison of the primary structures of thermophilic, mesophilic and psychrophilic lactate dehydrogenase (LDH) reveals a multitude of temperature-related amino acid substitutions. In the substitutions amino acid residues occurring preferentially in thermophilic, mesophilic (psychrophilic) LDH were found. On this basis, amino acid residues could be classified in an order from typical thermophilic (thermostabilizing) to typical mesophilic (thermolabilizing, increasing dynamics of the enzyme molecule) residues. The temperature-dependent ratio between thermostabilizing and thermolabilizing amino acid residues forms the basis for the specific structural and functional properties of thermophilic or mesophilic LDH. It is interesting that there appears to be a relationship between this order from thermophilic to mesophilic amino acid residues and the type of bases coding for these individual residues in the translation step of protein biosynthesis. Temperature-related amino acid substitutions are based on temperature-related base substitutions. A possible mechanism of temperature adaptation of LDH through alternative selection of thermophilic and mesophilic amino acid residues at the level of tRNA (anticodon)-mRNA (codon) interactions is discussed. These temperature-adaptation processes are evolutionary events in which the evolution and structure of the genetic code are involved.

1. Introduction

For all organisms temperature is a principal environmental factor. Temperature determines the rate of biological processes, the state of the structures of the proteins, nucleic acids and membranes, and the evolution of organisms and their cell components. During the course of evolution of organisms, cell metabolism, i.e., the structure and function of cell components, adapted to the environmental and cell temperature. Organisms are either thermophilic, mesophilic or psychrophilic when adapted to high, medium or low temperatures, respectively.

Correspondence address: H. Zuber, Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, ETH-Hönggerberg, CH-8093 Zürich, Switzerland.

Temperature is a particularly restrictive life condition. At high temperatures, for example, only microorganisms (bacteria) can exist. On the other hand, each organism has a relatively narrow temperature optimum for its metabolism, rarely exceeding 30 °C. In our research on the temperature adaptation of lactate dehydrogenase (LDH), we worked with thermophilic, mesophilic and psychrophilic bacilli. Each of these bacillary temperature variants (e.g., *Bacillus stearothermophilus, B. megaterium* and *B. psychrosaccharolyticus*) has a narrow temperature optimum within the particular temperature range (55–65, 40 and 25 °C). We can assume that under in vivo conditions the temperature optima of active cell components, enzymes for example, approach those of the bacilli themselves. The temperature optima of isolated thermophilic, mesophilic and psychrophilic LDH

shift under in vitro conditions, however, as the concentrations of both substrate and enzymes, and thus the concentration of the enzyme substrate complex, are different from the concentration in vivo. More representative in terms of temperature adaptation are the thermostabilities of LDH temperature variants (84, 45, 25°C).

What is the meaning of the temperature optimum of catalytic activity? On the one hand, the rate of enzyme-catalyzed reactions increases with rising temperature. On the other, inactivation of the enzyme or enzyme-substrate complex takes place, which leads to a drop in the activity curve with increasing temperature. A shift in the temperature optimum of LDH, e.g., from a thermophilic to a mesophilic type, brings about a change in thermostability and activity. Thermostability and activity are correlated. A thermophilic enzyme (LDH) is more thermostable at higher temperatures, but less active at lower temperatures than a mesophilic enzyme. Mesophilic and psychrophilic enzymes are more active at lower temperatures but less thermostable. Activity is determined by the dynamics of the enzyme molecule before and during the transition state of catalysis. In terms of energetics, dynamics is inversely proportional to the stability of the enzyme molecule. I may postulate here that the different temperature variants at their corresponding temperature optima are optimized to equal mobility in the transition state. Structural preconditions at the various temperature optima are different, however, due to the energetics of the active protein structure. Therefore, temperature adaptation of an enzyme, i.e., shifting of the temperature optimum, requires a long evolutionary process in which extensive structural changes (amino acid substitutions) take place.

What are these structural differences? What would we expect? Thermophilic, mesophilic and psychrophilic enzymes should differ in their amino acid sequence and protein conformation, i.e., ultimately in the number, strength and distribution of their non-covalent bonds. These weak bonds determine the stability and, on the basis of their dynamics, the mobility, i.e., activity, of the enzyme or enzyme-substrate complex. These bonds and their temperature dependence form the actual structural and energetic basis for the various temperature optima. With their double function (stability and directed dynamics), they form a delicately balanced network. Thus, one can expect that the shift of the temperature optimum among thermophilic, mesophilic and psychrophilic LDH will not be the result of large and extreme structural changes, but rather of a multitude of small changes, i.e., distributed amino acid substitutions, which are difficult to detect and interpret. Here the significant question arises as to whether analysis of these amino acid substitutions and their localization within the three-dimensional structure does in fact allow insight into the varying networks of non-covalent bonds in the temperature variants. The answer here is to a large extent a theoretical problem, but also a question of analytics, i.e., the determination of all amino acid substitutions related to temperature.

The differences in structure between the thermophilic and mesophilic enzymes are distributed over the entire molecule. Thus, all attempts to draw conclusions on the basis of individual amino acid substitutions only, i.e., without taking the whole molecule into account, have had little success. For example, the stabilizing role of single ion pairs or hydrophobic amino acid residues have been discussed. For this reason, comparative structural analysis of thermophilic, mesophilic and psychrophilic enzymes in which all amino acid substitutions were included became necessary. This yields a more complete picture of temperature adaptation in which not only the thermostabilizing role of certain amino acid residues but also their contribution towards temperature adaptation of activity (dynamics) is taken into account. This latter aspect of temperature adaptation does not generally receive due recognition.

2. Comparative primary structure analysis of thermophilic, mesophilic and psychrophilic LDH

We conducted such an extensive comparative structural analysis in the case of LDH from thermophilic bacilli (*B. stearothermophilus, B. caldotenax* and *B. caldolyticus*), mesophilic bacilli (*B. subtilis* and *B. megaterium*) and psychrophilic

bacilli (*B. psychrosaccharolyticus*) [1–7] (H. Zuber, manuscript in preparation). Thus, LDH temperature variants of phylogenetically related bacilli were compared in order to minimize general species differences in LDH structure determined by metabolism, which can conceal structural variations determined by temperature. The sequence homology is between 60 and 70%. LDH of bacilli is a tetrameric oxidoreductase (four identical subunits) which catalyzes preferentially the reduction of pyruvate to lactate. A good starting point for our investigations was the large body of functional and structural data obtained from muscle enzymes. Of particular importance are the high-resolution structural data on dogfish LDH (M. Rossmann and co-workers, J. Mol. Biol. (1987) in the press) which describe in detail the binding sites of the coenzyme, substrate. We compared the primary structures and the quality and quantity of amino acid substitutions (30–40% of the sequence substituted) including their positions within the three-dimensional structure. Total amino acid substitutions include both temperature related substitutions and other species-related substitutions. We assume that in the temperature adaptation of LDH in a cooperative system, temperature-related substitutions are overlapped by other substitutions, but in a specific way, and are therefore clearly discernible. We can further assume that the amino acid residues present in excess, i.e., the preferred amino acid residues in thermophilic, mesophilic and psychrophilic LDH, represent the typical thermophilic, mesophilic and psychrophilic amino acid residues. These are primarily responsible for the specific characteristics and properties of these temperature variants. It is further postulated that on the basis of the specific structure of these residues and their contribution to the stable or dynamic (active) protein structure, it is possible to classify these residues as more stabilizing and more destabilizing types. This classification is then valid for all amino acid substitutions, irrespective of whether or not they yield thermophilic or mesophilic residues in excess. It is therefore valid not only for the substitutions of thermophilic-mesophilic residues at the transition from thermophilic to mesophilic LDH, but also for the reverse substitutions of mesophilic-thermophilic residues at the transition from thermophilic to mesophilic LDH. It is difficult to judge whether these compensatory reverse substitutions are species-specific or determined by the mechanism of temperature adaptation. It remains to be answered, of course, to what extent this classification of amino acid residues in stabilizing and destabilizing types is dependent upon the type of the amino acid substitution and the position of these residues within the three-dimensional structure of LDH. For this reason it is important to relate the classification of the amino acid residue to the particular type of amino acid substitution. In our comparative structural analysis typical thermophilic, mesophilic and psychrophilic amino acid residues were therefore always determined in relation to a particular type of amino acid substitution. Here five groups of substitutions (including reverse substitutions) were considered: between hydrophobic-hydrophobic residues, charged-charged residues, hydrophobic-polar residues, charged-polar residues and polar-polar residues.

The primary structures of the LDH variants were compared pairwise in antisymmetric matrices. All LDH temperature variants were combined in this comparison. The total substitutions and reverse substitutions were recorded in the gross matrix. The net matrix contains all amino acid substitutions which result in preferentially thermophilic, mesophilic or psychrophilic residues and are not compensated by reverse substitutions. In the ideal case, i.e., when temperature-related substitutions are not overlapped by species differences, only substitutions and no reverse substitutions should remain. Thus, in the comparative structural analysis of the LDH variants, three series of data result: substitutions, reverse substitutions and substitutions compensated by identical reverse substitutions. From this, the type and number of amino acid residues selected for thermophilic, mesophilic or psychrophilic temperature variants can be derived.

What were the results obtained? With substitutions of hydrophobic for hydrophobic amino acid residues among the various temperature variants we find a clear splitting up between preferentially thermophilic Phe, Val and Ile residues and prefer-

entially mesophilic or psychrophilic hydrophobic residues Leu, Ala and Met in all combinations of the LDH temperature variants. Only a few reverse substitutions are present. The number of hydrophobic residues substituted corresponds to the difference in the temperature optimum or in thermostability among the LDH variants. Therefore, the number of temperature-related substitutions or preferred residues is small among the thermophilic LDHs (*B. stearothermophilus, B. caldotenax* and *B. caldolyticus*). It increases among the LDHs of *B. stearothermophilus* and *B. subtilis, B. megaterium* and *B. psychrosaccharolyticus*. Temperature-related substitutions with thermophilic and mesophilic hydrophobic residues also take place between the LDH of partly thermophilic *B. subtilis* and mesophilic or psychrophilic LDH of *B. megaterium* or *B. psychrosaccharolyticus*. Between mesophilic and psychrophilic LDH, however, they are compensated completely by reverse substitutions (species difference). On the basis of these data the hydrophobic residues may be arranged in the order from more thermophilic Phe, Val, via Ile, Leu to more mesophilic Ala, Met. The increased contribution of the more thermophilic residues at higher temperatures to stable and active thermophilic LDH structure lies in increased hydrophobicity, steric factors (packing density) and specific interactions (Phe, Tyr).

In the group of substitutions of charged-charged amino acid residues, the residues are also split up between preferred thermophilic residues (Arg, Asp) and preferred mesophilic residues (Glu, Lys). It is an interesting fact that in each category there is an acidic and a basic amino acid. This suggests that they are involved in ion pairs (e.g., thermostable Arg-Asp ion pairs or relatively mesophilic Glu-Lys ion pairs). But they might also be involved in hydrogen bonds with polar residues and/or with the NH or CO groups of the peptide bonds. When the primary structures of the different bacillary LDH are arranged within the three-dimensional structure of dogfish LDH, we find that an increased number of ionpairs, especially with Arg, is possible in thermophilic LDH, as compared to mesophilic LDH with more Lys. The ion pairs form stabilizing clusters, particularly in the region of the coenzyme-binding domain (contact area of subunits via the *Q*-axis, *R*-axis).

At the transition from thermophilic to mesophilic (psychrophilic) LDH, therefore, a restructuring of the network of hydrogen bonds takes place, in which charged residues are also involved. Included in this restructuring are substitutions of charged-polar residues and polar-polar residues. These are very detailed adaptations within the energetically balanced network of hydrogen bonds, in which steric factors and the polypeptide environment of the hydrogen bonds within the folded polypeptide or at the hydrated polar amino acid residues should play a significant role. It has been found that in the substitutions of charged-polar residues between thermophilic LDH (*B. stearothermophilus*) and mesophilic LDH (*B. subtilis* and *B. megaterium*) preferentially thermostable hydrogen-bond-forming charged residues are built into the thermophilic LDH. However, a large number of compensating reverse substitutions are found. The situation is more extreme in substitutions of polar-polar residues. However, there are also polar residues which for steric reasons predominate in thermophilic LDH (Asn) and those which predominate in mesophilic LDH (Gln). Interestingly, in some cases, Ser is preferentially built into thermophilic LDH, which can only be explained due to a particular steric situation. Charged residues and, in part, polar residues can be lined up, therefore, in an order from the more thermophilic Arg, Asp or polar Asn to more mesophilic Glu, Lys and polar Gln. The position of polar residues in this line-up, especially that of Thr, Ser, CysH, and His is, however, strongly dependent upon steric and energetic conditions of the hydrogen-bond system.

Essential and drastic substitutions at the transition from thermophilic to mesophilic (psychrophilic) LDH are that of hydrophobic-polar residues. Here is a clear predominance of temperature-dependent substitutions which result in specifically thermophilic, hydrophobic (particularly Ala) residues and in specifically mesophilic, polar (particularly Ser, Thr) residues. Here again there is a clear relation to temperature: practically no substitution (no preferred residues) is found either between thermophilic or mesophilic variants of LDH. In contrast, there are 12–13 substitutions

between thermophilic and mesophilic LDH. Virtually all of the substitutions lie within the LDH tetramer, most of them being partially or totally buried, and in strategically important regions: near the active site and in the contact regions of the subunits. Affected by the substitutions are the β-structure elements βA–D and βG–M and the helices αB, α1F, α2F, α3G, αH. Of special interest are the β structural elements βG, βH, and βK, βL, βM, which act as contact sites between the subunits over the *P*-axis or *R*-axis. Over the *Q*-axis the αB helix interacts with the αB helix and the α3G helix of the neighboring subunit, and the region between the αC helix and βC interact with α3G and α2F. Interestingly, the helix interaction system αB-α3G-α2F is in contact, or is part of, the active site (coenzyme, substrate-binding site). It lies in the center of the tetrameric molecule, around the central cavity. Amino acid substitutions of hydrophobic-polar residues in these structurally and functionally important regions should have an influence on both thermostability and dynamics (activity). The preferred thermophilic residues of thermophilic LDH should increase thermostability at high temperatures, but lead to a more rigid and less active structure at low temperatures. Polar residues (some possibly hydrated), on the contrary, should lead to a flexible and active structure at low temperatures, but labile structure at high temperatures. This may be one of the structural reasons for differing temperature optima of thermophilic and mesophilic LDH.

3. The significance of thermophilic-mesophilic (psychrophilic) amino acid substitutions for stable and active enzyme structure

Summarizing, the data from the comparative structural analysis of LDH allow us to derive the following view of structural differences between thermophilic and mesophilic (psychrophilic) LDH, including their significance for stable and active enzyme structure:

(1) In thermophilic LDH there is an increased portion of hydrophobic interactions and ion pairs increasing the free energy of the folded LDH molecule (more hydrophobic, charged amino acids, specific thermophilic hydrophobic, and specific thermophilic charged residues). This additional free energy serves on the one hand to stabilize and, on the other hand, to maintain the directed activity at higher temperatures (energetics of thermophilic catalysis).

(2) In mesophilic (psychrophilic) LDH, the greater number of polar residues results in an enlarged network of dynamic, i.e., weaker, hydrogen bonds. This leads, together with the hydrophobic interactions present, to optimum stability and directed dynamics (activity) at low temperatures. This type of adaptation holds even more for the low temperature region of psychrophilic LDH. Within this extreme temperature region, however, an increased number of charged residues (presumably stabilizing, perhaps through ion pairs) is also, for unknown reasons, necessary.

Thus, at the transition from thermophilic to mesophilic (psychrophilic) LDH, a restructuring takes place in which an energy-yielding portion of hydrophobic interactions and ion pairs is reduced in favor of the dynamic hydrogen-bond system. Conversely, at the transition from mesophilic (psychrophilic) to thermophilic LDH, dynamic bonds are converted to either thermostable hydrophobic interactions or ion pairs, or they are reinforced by these energy-yielding interactions.

With regard to the cooperative system of noncovalent bonds, in the restructuring of LDH among the diverse temperature variants, the temperature dependence of these bonds also plays a significant role. In the temperature range 20–80 °C, an increase in free energy of hydrophobic interactions and a decrease in free energy of the hydrogen bonds can be expected with rising temperature, while that of thermostable ion pairs (with Arg) should remain constant.

In summary, in all temperature variants of LDH the free energy of hydrophobic interactions and ion pairs should compensate for the conformation entropy, which also increases with temperature in order to guarantee a stable and active LDH structure prior to and in the transition state of catalysis. We can estimate on the basis of the difference in thermostability between thermophilic LDH (80 °C) and mesophilic LDH (50 °C) that in thermophilic LDH more free energy, of the order of

50 kcal/mol, is necessary to compensate for the increased conformation entropy. With respect to this value it is interesting to sum up all of the amino acid substitutions, insofar as they affect the increase in the energy-yielding, stabilizing hydrophobic interactions or ion pairs in thermophilic LDH as compared to mesophilic LDH. This balance shows that in all temperature variants compared the total number of these substitutions is correlated with thermostability. In the case of LDH from *B. stearothermophilus* and from *B. megaterium*, for instance, a total of 52 amino acid residues were substituted. If we assume a contribution of 1 kcal/mol free energy for each relatively stabilizing amino acid residue, this would result in 52 kcal/mol for the increased stabilizing energy of thermophilic LDH. On the average, approx. 15 amino acid substitutions would be necessary per 10 °C difference in thermostability. The temperature adaptation of LDH apparently took place in small steps during the course of evolution, whereby the substitutions, and compensating reverse substitutions, were distributed over the entire LDH molecule. This means that each partial region contributes to the specific properties of the temperature variants.

In order to assess the significance of the amino acid substitutions, their position within the three-dimensional structure is also important. It has been found that most of the temperature-related substitutions in the different temperature variants we compared lie at the same (or similar) sites of the primary structure and three-dimensional structure. Apparently there are regions of particular importance in temperature adaptation. This is especially crucial with regard to experiments currently in progress on directed mutagenesis of the diverse temperature variants of LDH. Knowing these specific regions, suitable mutants can be predicted better. The mutagenesis experiments aim to transform thermophilic (thermostable) LDH to mesophilic (psychrophilic) LDH mutants (and vice versa). This should allow us to check and confirm the findings of the comparative structural analysis of thermophilic and mesophilic amino acid residues.

4. Relationship between the temperature-dependent amino acid substitutions and the temperature-dependent base substitutions between thermophilic and mesophilic LDH

On the basis of the comparative structural analysis of the various LDH temperature variants the amino acid residues can be ordered both within the groups of hydrophobic, charged or polar residues and also between these groups with respect to their relatively stabilizing or destabilizing contribution in thermophilic or mesophilic LDH. It is interesting that there appears to be a relationship between this order, i.e., the type of amino acid residue, either thermophilic or mesophilic, and the type of bases coding for these residues. At first sight this is obvious for the amino acid residues which are coded by the third base of the codon. Relatively thermophilic residues such as Phe, Tyr, Asp and Asn are coded by C or U, relatively mesophilic residues such as Glu, Lys, Gln and Met by G or A. This indicates the possible existence of two types of tRNA selecting for C or U or G or A. Furthermore, there seems to be an assignment of hydrophobic, preferentially thermophilic residues to U and hydrogen bond forming, preferentially mesophilic amino acid residues (polar, charged residues) to A in the second base of the codon.

This relationship between preferred thermophilic or mesophilic amino acid residues and preferred bases coding for them in thermophilic or mesophilic LDH is revealed directly by comparison of the amino acid substitutions (or reverse substitutions) with the substitutions of the bases in the three positions of the DNA (mRNA) triplets of the thermophilic (*B. stearothermophilus*) and mesophilic (*B. megaterium*) LDH (H. Zuber, manuscript in preparation). The LDH genes of these bacilli have been cloned and sequenced [8,9]. Thermophilic LDH typically has a large number of C (21), U (12) plus G (10) and G (30) in the third, second and first base position of the codon. On the other hand, mesophilic LDH typically has much A in all positions of the codon triplet. The preference in the third base of the degenerate code for thermophilic C and mesophilic A corresponds to the specificity of the tRNA type for C or U or

G or A coding for preferentially thermophilic or mesophilic amino acid residues. A higher U content (and in part higher G content) in thermophilic LDH and a higher A content in mesophilic LDH in the second position of the codon correspond to a higher content of hydrophobic (thermophilic) and polar (mesophilic) residues, respectively. The substitutions G or A between thermophilic-mesophilic LDH in the first position of the codon is in part in accordance with the order within hydrophobic, polar and charged residues.

5. Preferred amino acid residues of thermophilic and mesophilic LDH are selected at the anticodon (tRNA)-codon level

These characteristic base substitutions between thermophilic and mesophilic LDH indicate two specific selection mechanisms active in temperature adaptation, one at the DNA level, the other at the polypeptide level. They are evidently coupled. The coupled selection mechanism affecting, for instance, the third base, became particularly apparent: (1) With respect to temperature adaptation of DNA/RNA, the C + G content in the third base of the codon is increased by 40% with respect to thermophilic LDH. It demonstrates that the structure of DNA/RNA has to adapt to temperature regarding a stable or a flexible (active) structure. (2) Temperature adaptation of protein structure (amino acid substitutions) on the basis of codon-anticodon interactions results in higher C content and a relatively high U content in the codon of thermophilic LDH (increase in C + U = 36%). In this selection process at the protein level not only the standard bases G-C and U-A pair, but also G-U. Interestingly, the G-U pairing shows a particular temperature dependence. On this basis it can be postulated that for protein temperature adaptation of LDH from bacilli either G (I) or U is preferentially used by the anticodons of tRNA of thermophilic or mesophilic LDH, respectively. Other microorganisms may use other types of tRNA with C or A in the first position of the anticodon, as for example extreme microorganisms like extreme thermophiles with C in the anticodon.

A direct relationship between the types of thermophilic or mesophilic amino acid residues and thermophilic and mesophilic bases substituted between thermophilic and mesophilic LDH is revealed by a detailed analysis of the individual bases in the codon of each amino acid residue. It shows that the characteristic order ranging from thermophilic to mesophilic amino acid residues corresponds to the specific order of bases ranging from thermophilic to mesophilic types in the three positions of the codon. For the first base this order is G-U-C-A, for the second, U-G-C-A and for the third, C-U-G-A. Thus, a similar (parallel) drift is observed in the substitutions of amino acid residues and bases from thermophilic to mesophilic types in the transition from thermophilic to mesophilic LDH.

In summary, these data on the relationship between directed amino acid and base substitutions of LDH demonstrate that a particular information content for an optimal enzyme structure which has been adapted in evolution and is still adaptable to various temperatures is contained in the triplet code. In this way the degenerate part of the code is also ordered. It is hypothetically assumed that tRNA, which carries a particular anticodon, is the coordinating point for directed adaptation of protein structure. The various, in part synonymous, tRNA molecules contain the information for amino acid residues, which either stabilize or mobilize the protein structure. The relative concentration of the tRNA species trigger positive selection. When the environmental temperature is changed, the relative concentrations of the tRNA molecules also change, initiating the search for more stabilizing or more mobilizing amino acid residues in mutants of the mRNA (DNA) by codon/anticodon interactions. By this selection process they transform mutations which occur on a statistical basis in DNA/RNA to accepted point mutations in an optimized protein structure. An excess of thermostabilizing or mobilizing residues will enhance the thermophilic or mesophilic properties, in particular by changing the balanced ratio between hydrophobic, charged and polar residues.

The first base of the anticodon G or U of tRNA pairs with C or U or G or A of the codon

in mRNA, respectively, thus selecting thermophilic and mesophilic residues, in a sense independently of the type of bases in the first and second position of the codon. This could indicate that the third base of the codon plays a leading role in temperature adaptation. It may be postulated that, both during evolution and today, temperature adaptation and modification of protein structures are initiated and directed by the third base of the codon. In many cases this would result in silent mutations, not apparent in the amino acid pattern. In these temperature-dependent silent mutations adaptative substitutions of the first and second base of the codon could be reinforced and directed. This role could be demonstrated by analyzing the third base substitutions between thermophilic and mesophilic LDH for such silent mutations. The silent mutations and base substitutions are concentrated on rather few amino acid residues. An extremely high substitution rate is found in the case of Val, Ala and Gly (G as the first base of the codon) and a relatively high substitution rate with amino acid residues coded by U (hydrophobic residues) or, in part by A and C (polar, charged) of the second base of the codon. Importantly for the substitutions of the third base, the characteristic drift thermophilic-mesophilic bases can again be recognized in the order C-U-G-A for the transition between thermophilic and mesophilic LDH. The data demonstrate that in silent mutations C or U are also preferentially selected in thermophilic LDH and G or A in mesophilic LDH. This seems to be particularly important for Val, Ala and Gly and hydrophobic amino acids in general, which for energetic and probably phylogenetic reasons seem to be the starting point to vary adaptatively an active protein structure. From this point, with a high substitution rate, the temperature-related amino acid substitutions are originating to yield either thermophilic or mesophilic amino acid residues, e.g., by one-base amino acid substitutions of the first or second base. In this way not only hydrophobic, but also polar and charged amino acid residues are selected, adapting the important balanced ratio of hydrophobic interactions-hydrogen bonds. These results on the selective power of the third base of the codon show that the degenerate code has a specific logic, i.e., information content. This logic is connected with the structure and evolution of the genetic code.

6. Concluding remarks on the structure and evolution of the genetic code and on the general significance of the temperature adaptation data of LDH

The combined information content of the bases in the three positions of the codon/anticodon selective for optimum protein structures adapted to different temperatures is stored in the structure of the genetic code. The combined drifts between relative thermophilic and relative mesophilic bases in the three positions of the codon can be correlated to the individual relative thermophilic or mesophilic amino acid residues. A more detailed understanding of the code structure with respect to thermophilic or mesophilic residues is obtained on the basis of the evolution of the code. On the other hand, on the basis of these coupled temperature adaptation processes at the protein and DNA/RNA level, general principles of the evolution and structure of the genetic code correlated to the evolution of the proteins and nucleic acids, can be deduced. In this respect, the following aspects are important:

(1) In the evolution of proteins the balanced ratio between hydrophobic interactions, ion pairs and hydrogen bonds is developing continuously depending on the evolving amino acid pattern (type of amino acid available) and on the environment.

(2) Parallel to the evolution of the proteins, there was the evolution of the adaptive codon/anticodon system, i.e., the tRNA adapter molecules with their specific information content for specific amino acid residues with specific properties. This development is coupled with the evolution of RNA/DNA.

(3) On the basis of these two processes (items 1 and 2), a symmetric code structure developed which still contains the information for optimum protein structures and the adaptive codon/anticodon system. The theory describing this interpretation and evolution of the genetic code ('Symmetry Theory') is described elsewhere (H. Zuber, manuscript in preparation).

Finally, of course, the question which remains is whether these data of the temperature adaptation of LDH and the considerations on the positive selection processes on the basis of a specific code structure also hold for the temperature adaptation of other enzymes or proteins and, in general, for proteins which must adapt to changing environmental conditions (ion concentration, pH). In all these cases the ratio between the hydrophobic interactions and ion pairs and the dynamic network of hydrogen bonds is affected. We expect to find similar mechanisms of positive selection and adaptation also here.

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