Molecular Basis for the Transfer of Nicotinamide Adenine Dinucleotide among Dehydrogenases†

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ABSTRACT: NADH is transferred directly from one dehydrogenase enzyme site to another without intervention of the aqueous solvent whenever the two dehydrogenases are of opposite chiral specificity as regards the C_4 H of NADH which is transferred in the catalyzed reduction reaction. When both enzymes catalyze the transfer of hydrogen from the same face of the nicotinamide ring, direct enzyme–enzyme transfer of NADH is not possible [Srivastava, D. K., & Bernhard, S. A. (1984) *Biochemistry 23*, 4538–4545; Srivastava, D. K., & Bernhard, S. A. (1985) *Biochemistry* (preceding paper in this issue)]. Utilizing an advanced computer graphics facility, and the known three-dimensional coordinates for three dehydrogenases, we have investigated the feasibility of various aspects of the direct transfer of dinucleotide from the site of one enzyme to the site of the other. The facile passage of the coenzyme through the first enzyme site requires an open protein conformation, characteristic of the apoenzyme rather than the holoenzyme structure. Since two dehydrogenases of the same chirality bind coenzyme in the same conformation, the direct transfer of coenzyme from one site to the other is impossible due to the restriction in molecular rotation of the coenzyme in the path of transfer from one binding site to the other; therefore, coenzyme can only be transferred from one dehydrogenase site to another site via the intermediate dissociation of coenzyme into the aqueous milieu. In contrast, when an A dehydrogenase and a B dehydrogenase are juxtaposed, it is stereochemically feasible to transfer the nicotinamide ring from its specific binding site in one enzyme to the site in the other. Dissociation of the first enzyme from the rest of the coenzyme molecule permits an internal rotation of the nicotinamide about the glycosidic bond by 180°. This permits the entry of the rest of the coenzyme molecule into the second enzyme binding site in proper conformation for specific interaction with the second enzyme protein. A calculation of the electrostatic potential molecular surface of the enzyme face containing entry to the coenzyme binding site of the three dehydrogenases of known structure reveals that the two A dehydrogenases have large areas of negative potential, whereas the B dehydrogenase has large areas of positive potential, on the surface around the opening to the nicotinamide end of the binding site. Thus, the structural information available is consistent with the observed exclusive direct transfer of coenzyme between dehydrogenases of opposite chirality.

The classic work of Westheimer and his collaborators (Fisher et al., 1953) demonstrated the stereospecific transfer of hydrogen between the C_4 position of the nicotinamide ring and substrate (Pullman et al., 1954; Arnold et al., 1976). These findings have been widely extended to other NAD^+-dependent dehydrogenases (see Simon & Kraus, 1976, You et al., 1978, You, 1982, and references cited therein). The stereospecific transfer of hydrogen is a ubiquitous feature of NAD^+-dependent dehydrogenases. Two classes of NAD^+-dependent dehydrogenases have been identified (A and B); they differ in which of the two C_4 hydrogens of NADH is transferred during the reaction (Cornforth et al., 1962).

Recently, it has been demonstrated that the direct transfer of metabolites from one enzyme to another may proceed without the intermediate intervention of the aqueous solvent (Weber & Bernhard, 1982; Srivastava & Bernhard, 1984, 1985). Similar but less definitive results were presented much earlier, indicative of the direct transfer of NADH from one dehydrogenase to another, for example, from glyceraldehyde-3-phosphate dehydrogenase (GPDH)[1] to either liver alcohol dehydrogenase (LADH) or lactate dehydrogenase (LDH) (Cori et al., 1950; Nygaard & Rutter, 1956). In the preceding paper (Srivastava & Bernhard, 1985), we present evidence that this direct transfer mechanism, via an enzyme–coenzyme–enzyme complex, is not ubiquitous. The consistent pattern in these results is that A dehydrogenases can transfer NADH directly to B dehydrogenases and vice versa but that A–A and B–B transfers can only occur via the intermediate dissociation of NADH into the aqueous solvent (Srivastava & Bernhard, 1984, 1985).

The determination of the three-dimensional structures of a number of dehydrogenases to atomic resolution has presented a satisfactory phenomenological explanation for the stereospecific transfer of hydrogen in the NAD^+-dependent dehydrogenases (see Rossmann et al., 1975, 1977, Brändén & Eklund, 1980, Grau, 1982, and references cited therein). A-face dehydrogenases (LADH, LDH, and malate dehydrogenase) interact with coenzyme in a similar fashion

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[1] Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase; LADH, horse liver alcohol dehydrogenase; LDH, lactate dehydrogenase; α-GDH, α-glycerolphosphate dehydrogenase.

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(Brändén et al., 1975; Holbrook et al., 1975; Banaszak & Bradshaw, 1975), and in a way which is different from the interaction of the coenzyme with a B dehydrogenase (GPDH) (Buehner et al., 1974; Moras et al., 1975; Olsen et al., 1976; Biesecker et al., 1977). Invariably, the coenzyme interacts with protein in such a way that only one face of the dehydrogenase is exposed in the direction of the catalytic site for substrate. The tight interaction of the protein with one face of the nicotinamide and the exposure of the opposite face toward the catalytic domain define the A vs. B chiral specificity (see discussion below).

Given our experimental results on the chiral transfer of NADH between pairs of dehydrogenases, and the known three-dimensional structures to atomic resolution for three dehydrogenases (two A dehydrogenases and one B dehydrogenase), it appeared to us that a molecular explanation for the chiral specificity might be at hand. Toward this end, we have investigated the molecular aspects of transfer utilizing the computer graphic facilities at the University of California Medical Center in San Francisco (Langridge et al., 1981). The first objective of this investigation was to ascertain the feasibility of bringing the two dehydrogenase molecules into proximity, so as to effectively exclude the solvent environment, without the introduction of strong repulsive interactions. Given the success in this first objective, the question arises as to whether the stereospecific transfer of coenzyme in this pathway is possible. Results and conclusions from these studies are contained in the following sections.

The analysis which we present herein tests the possibility of stereospecific coenzyme transfer within an enzyme–enzyme complex, among the three dehydrogenases of known structure.

Materials and Methods

Computer graphic analyses were carried out by utilizing coordinates[2] for the binary coenzyme complex of lobster GPDH (Buehner et al., 1974; Moras et al., 1975) and the ternary substrate–coenzyme complex of pig LDH (Grau et al., 1981) and horse LADH (Cedergren-Zeppezauer et al., 1982). The tetrameric structures for GPDH and LDH were generated by rotations of the published coordinates around the P, Q, and R axes (Buehner et al., 1974; Grau et al., 1981). The dimeric structure of LADH was generated by superimposing subunit coordinates, by the least-squares method, onto residues 306–309 of the second subunit which was included in the data set.

Electrostatic potential molecular surfaces were calculated according to Weiner et al. (1982). The calculation was done by first determining the solvent-accessible surface and surface normals with the program MS (Connolly, 1983). Since the transfer of coenzyme involves an intermolecular site–site interaction, only the surface on the side of the enzyme containing the binding cleft was calculated. The surface did include, however, contributions from both the catalytic and binding domains. The potential was then calculated from the classical formula, at a distance of 1.4 Å outside the surface along the surface normal, for each point (Weiner et al., 1982). Partial atomic charges, derived at pH 7.0 by quantum mechanical calculations (Singh & Kollman, 1984), were assigned to heavy atoms about polar hydrogens in each amino acid in the data set. A distance-dependent dielectric constant was used throughout the calculations (Hopfinger, 1973; Gelin & Karplus, 1979). Coordinates for NAD^+ were not included in the calculation. Since NAD^+, as well as NADH, is transferred directly between A and B dehydrogenases (D. K. Srivastava and S. A. Bernhard, unpublished results), the electrostatic contribution of the nicotinamide charges cannot be crucial to the conclusions. For GPDH and LDH, the calculation included contributions from atoms within 30 Å of each point. In GPDH and LDH, where there is no apparent specific cation ligation, the method of neutral spheres was used to reduce electrostatic effects due to the 30-Å cutoff. Atoms within 60 Å, which include the two specific Zn ligands per subunit, were included in the calculation with LADH, and the method of neutral spheres was not used. The data set for each dehydrogenase included the coordinates of the holoenzyme structures for one subunit including residues from the adjacent subunit: residues 306–309 from LADH, residues 60–73 from LDH, and residues 183–189 from GPDH.

Skeletal models and electrostatic potential molecular surfaces for the enzymes were displayed and manipulated on an Evans and Sutherland picture System 2 with the program MIDAS (Huang et al., 1983). Pairs of oligomeric enzymes were manipulated independently of each other to determine possible configurations for two enzymes in intimate contact. To consider the stereochemistry of the coenzyme transfer, while searching for paired enzyme configurations, the holoenzyme structures were displayed rather than one apo structure and one holo structure as the transfer process would dictate. In crystallographic determinations utilized herein, either the apo structures are of considerably lower resolution or there is no apo structure determination.

Results and Discussion

Our primary objective in this paper is to consider molecular pathways which explain the observed chiral specificity in the direct transfer of NADH from one dehydrogenase site to another (Srivastava & Bernhard, 1984, 1985). The initial simplest approach toward this problem is to assume that at some time the surfaces around the coenzyme binding clefts of the two dehydrogenases are in intimate contact. Three criteria must be met in order for transfer to occur in this situation. (1) There should not be any large steric or electrostatic repulsion as a consequence of the juxtaposition of the two enzymes. (2) The coenzyme molecule must be capable of free passage out of the first binding site and into the second binding site. There must be no large energy barrier restricting the translational motion of the coenzyme molecule in this direction. (3) Since the coenzyme–protein interaction in the dehydrogenase site involves specific enzyme–coenzyme interaction and specific coenzyme conformation, the passage of coenzyme from one site to the other must allow the capability for molecular rotation and/or internal rotations of the coenzyme molecule in order to meet the stereospecific complementary demands of the protein structure of the second site.

In order to consider the direct transfer process, it is first necessary to consider in more detail some of the properties of the individual enzymes and of their coenzyme complex structures. In addition to the steric constraints arising from van der Waals and hydrogen bonding interactions between the two three-dimensional structures, the electrostatic potential of each of the pairs of interacting surfaces is of consequence. It is also essential to consider the possibility of movement of the coenzyme molecule within each of the protein structures before examining the process of direct transfer from one site to another.

Electrostatic potential molecular surfaces were calculated for the enzyme surfaces around the coenzyme binding cleft.

[2] GPDH and LDH coordinates were obtained from the Brookhaven Protein Data Bank. Coordinates for LADH were received from Dr. E. Cedergren-Zeppezauer.

The result of the calculation was displayed on the computer graphics picture system, by color coding the surface points according to electrostatic potential (Weiner et al., 1982). In this way, it was possible to view the negative, neutral, and positive potentials of the surface around the coenzyme binding cleft. Since the composite surface potential is difficult to illustrate, we have displayed negative ($E < -4$ kcal/mol), neutral ($E > -4E < 4$ kcal/mol), and positive ($E > 4$ kcal/mol) regions for three dehydrogenases (LADH, LDH, and GPDH) in the supplementary material (see paragraph at end of paper regarding supplementary material available). The composite surface potential can be reconstructed by superposition of the figures in the supplementary material. Alternatively, more detailed surfaces can be provided by one of us (J.A.M.) upon request. The two A-type enzymes (LADH and LDH) both have a predominantly negative molecular surface above the nicotinamide ring which forms the entrance cleft to the ring binding site. Negative surface potentials were found for LADH around the residues 10–26 and 44–64 in the catalytic domain, and near residue 297 on the other side of the cleft. The potentials around the residues 218–250, 195–203, and 310–330 of the catalytic domain of LDH were negative, as well as part of the flexible loop around residue 107. GPDH, the only B dehydrogenase of known structure, exhibited a very different contour pattern for the electrostatic potential molecular surface. The surface around the nicotinamide binding cleft was found to be more positive. In particular, the residues in the catalytic domain, 173–231, and residues 123, 98, and 183 from the adjacent subunit exhibited a predominantly positive potential.

An examination of the holoenzyme structures of all three dehydrogenases reveals that a substantial part of the coenzyme molecule, particularly the nicotinamide ring which is deeply buried, is inaccessible from the outside surface (Rossmann et al., 1975; Holbrook et al., 1975; Harris & Waters, 1976; Bränden & Eklund, 1980). Nevertheless, the coenzyme is capable of dissociation from the enzyme site, and the subsequent reassociation in aqueous solution occurs with a specific rate constant not substantially lower than that associated with free diffusion (Gutfreund, 1975; Hammes, 1982). Presumably, the holo → apo transition allows for the free passage of the coenzyme out of the binding sites of all the dehydrogenases.

An examination of the apoenzyme structures shows that in each case the nicotinamide end of the coenzyme binding cleft is more open to the exterior than in the holoenzyme structures. The catalytic domain of LADH rotates about 7° around a hinge region when the coenzyme is complexed, closing the coenzyme cleft (Eklund et al., 1981). Similarly, the effect of coenzyme binding to GPDH is to move the two domains closer together (Murthy et al., 1980). LDH also undergoes a conformational change upon coenzyme binding (White et al., 1976). A flexible loop consisting of residues 98–118 is in the open position in apo-LDH. In the holoenzyme, this sequence forms a part of the coenzyme binding site (White et al., 1976). Although the global changes in conformation of the proteins are not large between holo- and apoenzyme, there are substantial local changes in conformation, particularly in the surface region surrounding the opening to the nicotinamide end of the coenzyme binding cleft. These particular surfaces are the ones by which the docking of cognate enzymes is realized. Since the apoenzyme structures are in all cases less refined than the holoenzyme structures, and since we have no direct evidence that pure apo conformations are required for the direct transfer of coenzyme between dehydrogenases, there is necessarily a substantial degree of imprecision in the docking process which we shall describe below.

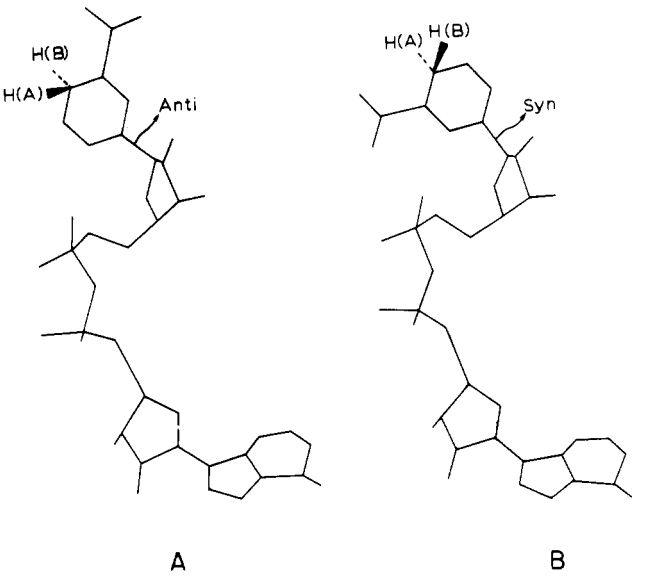

FIGURE 1: Conformation of NADH when bound to A (left) and B (right) dehydrogenases. The designations anti (left) and syn (right) refer to the orientation of the nicotinamide ring relative to that of the ribose. A dehydrogenases interact with coenzyme so as to leave only the H(A) hydrogen at C_4 accessible to small-molecule substrate, whereas B dehydrogenases interact with the opposite face of the nicotinamide ring so as to leave only H(B) exposed to substrate. The protein–coenzyme complementarity is illustrated schematically in Figure 2.

In each of the dehydrogenases, specific polar and hydrophobic interactions between the enzyme and the coenzyme exist. These tight hydrophobic and polar interactions between side chains of the protein and various molecular groups of the coenzyme restrict the internal rotation of the coenzyme when bound (see Rossmann et al., 1975, Brändén & Eklund, 1980, Grau, 1982, and references cited therein). Utilizing the atomic coordinates for each of the three dehydrogenases of known structure, it is impossible to rotate the nicotinamide moiety about the glycosidic bond by ~180° so as to expose the opposite face of the nicotinamide to the catalytic domain of the protein. Thus, the conformation is rigorously specified by its particular interaction with the dehydrogenase.

There are two basic conformations of the dehydrogenase-bound coenzyme as illustrated in Figure 1. In the A-type dehydrogenase, LDH and LADH, the conformation about the nicotinamide N_1–C_1' glycosidic bond is anti. The B hydrogen is projecting toward a protein surface, and the A hydrogen is projecting toward the substrate binding site. A rotation of about 180° around this glycosidic bond produces the syn conformer found in the B dehydrogenase. Although the binding sites for A and B dehydrogenases are similar, they are quite specific for either the anti or the syn conformer, respectively (Rossmann et al., 1975; Simon & Kraus, 1978; Brändén & Eklund, 1980; Grau, 1982). The specificity is due to the specific interactions between the protein and the carboxamido group in each case (Holbrook et al., 1975; Garavito et al., 1977; Cedergren-Zeppezauer et al., 1982). It is interesting that nature has apparently selected to unambiguously identify the anti conformation of the coenzyme with the A dehydrogenase and the syn conformation of the coenzyme with the B dehydrogenase. We shall return to this structural feature in our discussion of the chiral transfer of coenzyme. It is also interesting to note that although the torsion angles are in general fairly similar, they need not be the same for coenzyme bound to different dehydrogenases of the same chirality (Grau, 1982; Gronenborn & Clore, 1984). For example, the torsion

angles for NAD^+ bound to sorbitol dehydrogenase are considerably different from those found in LADH (Gronenborn & Clore, 1984). Nevertheless, the anti conformation about the nicotinamide glycosidic bond and the open structure exposing one face of the nicotinamide ring are evident. What is maintained constant among dehydrogenases of the same chirality is the specific protein interaction with one face of the nicotinamide and the exposure of the opposite face to the catalytic domain.

In the following comparison of structures, we assume explicitly, as others have assumed implicitly, that the general conformation of the protein and of the coenzyme is the same in the enzyme–NADH complex as it is in the enzyme–NAD^+ complex (see Banaszak & Bradshaw, 1975, Holbrook et al., 1975, Rossmann et al., 1975, Brändén & Eklund, 1980, and references cited therein). In the case of LADH, where the structures of oxidized and reduced coenzyme complexes have been determined, this assumption has been confirmed to a level of resolution sufficient for the following discussion (Brändén & Eklund, 1980; Eklund et al., 1981; Cedergren-Zeppezauer et al., 1982). Moreover, in experiments we shall report on subsequently, the direct transfer of NAD^+, as well as NADH, between enzymes of opposite chiral specificity has been demonstrated (D. K. Srivastava and S. A. Bernhard, unpublished results).

Possible configurations of pairs of dehydrogenases were tested by computer graphics. It was possible to arrange the enzymes so that the coenzyme binding clefts were facing each other, without introducing serious steric repulsions. Given the stereospecific nature of the transfer process, juxtaposition of the enzyme surfaces immediately in front of the buried nicotinamide ring seemed most likely. It is possible to bring the enzymes together in this manner so that one enzyme site from each of the two oligomeric dehydrogenases is involved in the interaction. In the case of A–B enzyme pairs, the nicotinamide rings are mirror images, and in A–A or B–B enzyme pairs, the nicotinamide rings are rotated by 180° with respect to each other. The adenine termini of the coenzymes are always related by a 180° rotation when the two sites are facing each other as shown in Figure 2. The exterior surfaces of the enzymes directly in front of the buried nicotinamide ring and around the opening to the binding cleft are in close contact. In LADH, these surfaces are around residues 10–26 and 44–64 in the catalytic domain and residue 297 in the coenzyme binding domain. In LDH, the surfaces include residues 218–250 in the catalytic domain and 105–108 of the flexible loop. Residues 173–231 in the catalytic domain and 96–127 in the binding domain of GPDH form part of the interacting surface. When the two interacting enzymes are of opposite chirality, their surface electrostatic potentials are of opposite sign, and consequently, such interactions are favored electrostatically in addition to the many favorable van der Waals interactions among the atoms of the interacting surfaces. Hence, all criteria are met for a "docking" of two dehydrogenases of opposite chiral specificity. However, we have not ruled out the possibility of transfer of coenzyme between two enzymes of the same chirality on the basis of electrostatic surface potential alone. The magnitude of the electrostatic energy of interaction is determined by a variety of environmental factors. These factors include the pH of the medium, the dielectric constant for the "solvent" surrounding the interacting charges, the ionic strength, and the dependence of the dissociation constants of the weak acids and bases of the protein constituents (Harned & Owen, 1958). Our interest in electrostatic surface potentials is only to ascertain the

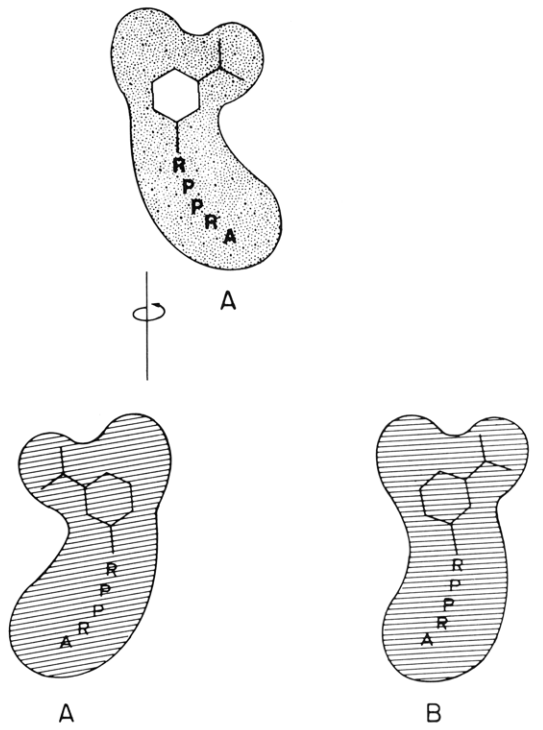

FIGURE 2: Schematic representation of transfer of NAD^+ between a pair of dehydrogenases oriented with the cleft entries juxtaposed. The coenzyme conformations when bound to A and B dehydrogenases are indicated. Coenzyme is indicated as occupying all three dehydrogenase sites so as to illustrate the problem of coenzyme transfer between two A dehydrogenases (lower left complex to upper complex) and two dehydrogenases of opposite chiral specificity (lower right complex to upper complex). The protein–coenzyme complementarity is also indicated by the shaded binding surface. The outside surface of the protein opposite to the cleft entrance is indicated by the lined surface. The symmetry operation for a pair of identical A dehydrogenases is indicated between the lower left complex and the upper complex.

feasibility of bringing two such surfaces together. Such juxtaposition is clearly feasible when the two dehydrogenase surfaces are contributed by enzymes of opposite chiral specificity.

We pose two structural problems. (1) Can coenzyme in one conformation be transferred directly from one dehydrogenase to a second apodehydrogenase of the same coenzyme chiral specificity? (2) Can coenzyme be transferred directly from a dehydrogenase of one specificity to a second apodehydrogenase of opposite chiral specificity? In either case, the coenzyme must assume a conformation complementary to the new binding site while either exiting from protein 1 or entering protein 2.

Transfer of Coenzyme between the Sites of Same Chiral Specificity. In the case of the A–A enzyme pair (or GPDH–GPDH, B–B pairs), the two coenzyme binding clefts can be brought together, albeit with some degree of unfavorable electrostatic interactions. In order for the coenzyme to transfer from site 1 to site 2, it must effect a molecular rotation of 180° as illustrated in Figure 2. There is no entrance to the binding sites where the coenzyme can rotate while either leaving one enzyme cleft or entering the second enzyme cleft. The adenine binding pocket, which is on the surface and which is essentially preformed in all three dehydrogenases, maintains the orientation of the coenzyme (see Rossmann et al., 1975, Brändén & Eklund, 1980, Grau, 1982, and references cited therein). Therefore, it is clear that transfer of coenzyme between dehydrogenases of the same chirality necessarily involves the intermediate dissociation of coenzyme from the first site into

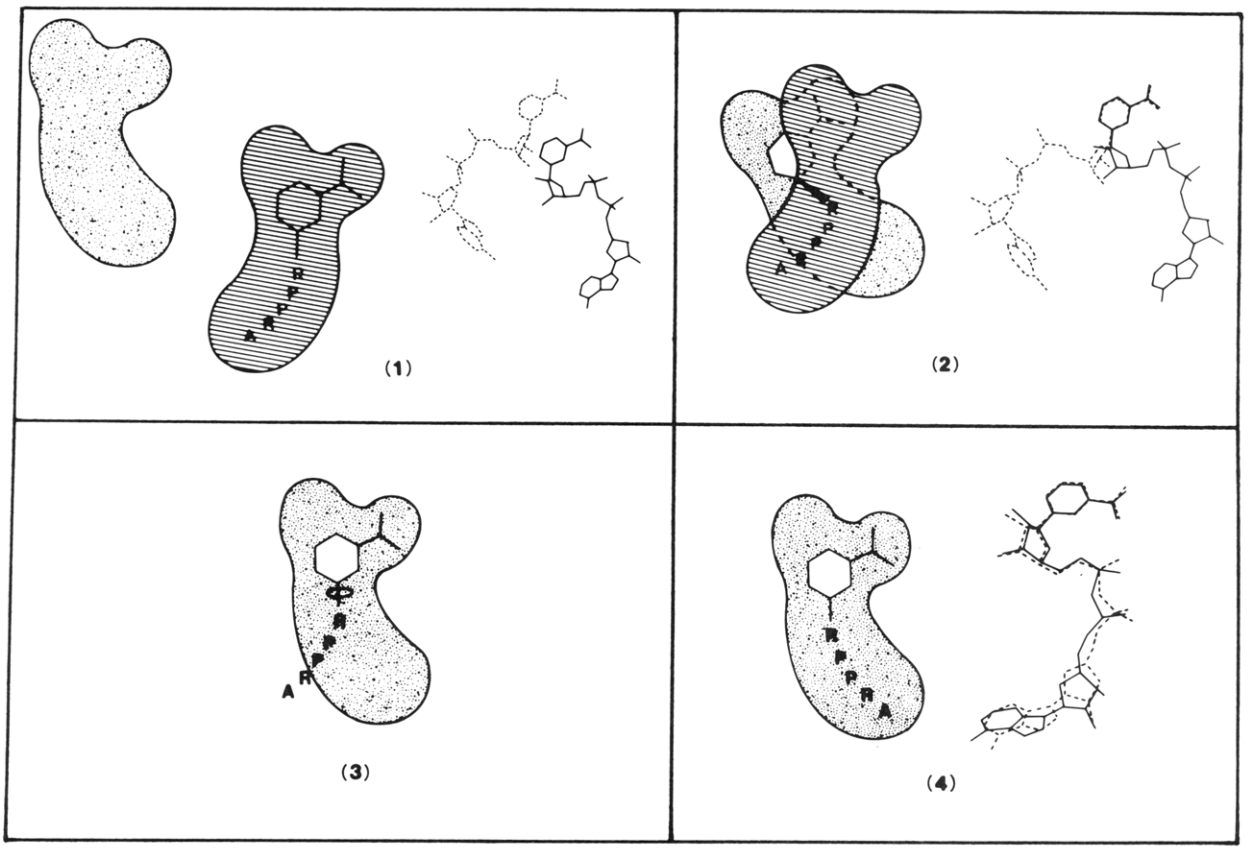

FIGURE 3: Mechanism of direct transfer of NAD^+ between a pair of dehydrogenases of opposite chirality. The transfer of NAD^+ from a B dehydrogenase site (lower) to an apo A dehydrogenase (upper) is illustrated. Shaded and lined surfaces are as in Figure 2. The sequence of events is schematized as follows. (1) The two enzyme sites are oriented "cleft to cleft" as in Figure 2. (2) The nicotinamide ring is transferred from site B to site A. Note the impossibility of transfer of the dinucleotide structure by translation alone. (3) The front (B) dehydrogenase dissociates from the rest of the coenzyme, allowing free rotation about the nicotinamide N_1–C_1' glycosidic bond. (4) Following rotation of ~180° about the N_1 glycosidic bond, the rest of the dinucleotide structure can bind to the A dehydrogenase site by translation into the second cavity. The *precise* geometry of the coenzyme in (1), (2), and (4) taken from the computer graphics for the docking and transfer of NAD^+ from holo-GPDH to apo-LADH are shown along with the schematic view of the enzyme–coenzyme complexes. The solid and dotted lines are the conformations for the coenzymes as they appear in the holo-GPDH and LADH structures, respectively.

the solvent environment before it can become accessible to the second enzyme site.

Transfer of Coenzyme between the Sites of Opposite Chiral Specificity. If we examine the transfer of the coenzyme between sites of opposite chirality, the situation is different. The proteins can approach each other so as to make good van der Waals contact with the exclusion of solvent. Since the electrostatic potential surrounding the upper part of the GPDH site is positive, opposite to that for the A dehydogenases (LADH and LDH), it is possible to have a favorable electrostatic interaction. When the sites of two dehydrogenases of opposite chirality are juxtaposed, then a coenzyme molecule bound to one site can transfer its nicotinamide to a second structurally complementary site by translation alone as illustrated schematically in Figure 2. We have made such a juxtaposition so that the cleft openings are superimposed. Utilizing computer graphics, it is possible to make such a juxtaposition so that the nicotinamide ring can be transferred in proper orientation for binding to the nicotinamide site of the enzyme of opposite chirality.

The docking procedure is such that the two coenzyme binding sites are oriented as illustrated in Figure 3. It proved impossible herein to illustrate the docking of the two proteins. The specific mode of docking is defined, however, by the orientation of the two coenzyme sites (Figure 3), by the published coordinates for holoenzymes, and by the locations of specific surface amino acid residues at the cleft entries. We are at present refining the docking restrictions and the graphic representation and hope to report on a higher resolution and more restricted docking situation in a future publication. In the discussion which follows, we represent the proteins schematically. We do, however, give actual computer graphic representations for the coenzyme transfer processes (Figure 3).

Following the juxtaposition as discussed above, the nicotinamide ring can dissociate from a B enzyme site and bind directly to an A enzyme site as illustrated in Figure 3. Alternatively, the nonpolar nicotinamide ring of an unassociated holoenzyme becomes exposed to solvent by partial exit from the site; at this time, the second enzyme site interacts so as to relieve the unfavorable hydrophobic interactions between the nicotinamide ring and solvent [Figure 3(2)], leading to the same juxtaposition as described above. This recognition of the nicotinamide ring allows for a partial anchoring to the second site. Note in this case that the adenine portion of the coenzyme is not in proper position vis. à vis. the A dehydrogenase site to be complementary. The constraint of the enzyme cleft prohibits internal rotation of the rest of the coenzyme molecule while it is bound to the first enzyme site. To achieve a complementary conformation, the residual coenzyme must first dissociate from the first binding site [Figure 3(3)]. The dissociation allows for a rapid internal rotation of 180° about the nicotinamide N_1–C_1' glycosidic bond and the entry of the rest of the dinucleotide into site 2 with a conformation appropriate to the transfer of hydrogen with opposite chirality [Figure 3(4)]. There is a low barrier to

rotation in the aqueous solvent, and it should therfore be facile.

It may not be intuitively obvious why we have chosen to examine the coenzyme transfer starting with the nicotinamide end of the dinucleotide. An examination of the holodehydrogenase structures shows that the adenine end of the molecule is bound in the same way regardless of the chirality of hydrogen transfer. Hence, by aligning the cleft openings of any pair of dehydrogenases, it is not possible to transfer the adenine end from one cleft to another by means of translations alone (Figure 2). Assuming that the structures obtained crystallography relate to the coenzyme transfer processes which we have observed in aqueous solution (Srivastava & Bernhard, 1985), direct transfer can only occur via the initial transfer of the nicotinamide end of the dinucleotide.

It is interesting to note that the direct transfer of NADH among dehydrogenases of opposite chiral specificity is always facile. For example, the minimal V_{max} for the reduction of pyruvate by GPDH–NADH catalyzed by LDH is at least 700 s^{-1} (Srivastava & Bernhard, 1985). The V_{max} for NADH transfer from LDH to either GPDH or α-GDH is fast enough to allow a V_{max} of several hundred per second, a rate comparable to the maximal velocity obtained by utilizing aqueous NADH (Srivastava & Bernhard, 1984, 1985). These numbers demonstrate that the rate of NADH transfer from enzyme to enzyme far exceeds the measured "off" rates of NADH into the aqueous solvent (see Holbrook et al., 1975, Harris & Waters, 1976, Brändén et al., 1975, and references cited therein). Such dissociation rates are typically in the range of 10–100 per s. Hence, it might appear that the direct transfer process catalyzes the off rate for NADH from the carrier enzyme. An alternative explanation for this phenomenon is that the docking of the two proteins removes an inhibition on the dissociation of NADH from the carrier enzyme. This possibility is plausible since the dissociating nicotinamide ring would probably be forced back into the enzyme crevice by the interaction of solvent molecules with a relatively nonpolar end of the nicotinamide residue. If docking proceeds with the exclusion of solvent, as appears feasible from the computer graphics juxtaposition, the motion of the nicotinamide may be considerably more dynamic; the modest free-energy change predicted for the partial dissociation of the nicotinamide moiety from the specific site is compatible with a more rapid dissociation rate.

The experimental results we have presented on the transfer of NADH between dehydrogenases of opposite chirality demonstrate unambiguously that direct transfer via an enzyme–enzyme complex occurs (Srivastava & Bernhard, 1984, 1985). The present analysis shows that such a complex must at best be metastable in order that the complete transfer can take place.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Figures showing negative, neutral, and positive regions of the three dehydrogenases LADH, LDH, and GPDH (13 pages). Ordering information is given on any current masthead page.

Registry No. NAD, 53-84-9; GPDH, 9001-50-7; LDH, 9001-60-9; LADH, 9031-72-5; dehydrogenase, 9035-82-9.

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Specific Arginine Modification at the Phosphatase Site of Muscle Carbonic Anhydrase†

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ABSTRACT: Mammalian carbonic anhydrase III has previously been shown to catalyze the hydrolysis of *p*-nitrophenyl phosphate in addition to possessing the conventional CO_2 hydratase and *p*-nitrophenylacetate esterase activities. Modification of pig muscle carbonic anhydrase III with the arginine reagent phenylglyoxal yielded two clearly distinctive results. Reaction of the enzyme with phenylglyoxal at concentrations equivalent to those of the enzyme yielded stoichiometric inactivation titration of the enzyme's phosphatase activity, approaching 100% loss of activity with the simultaneous modification of one arginine residue, the latter based on a 1:1 reaction of phenylglyoxal with arginine. At this low ratio of phenylglyoxal to enzyme, neither the CO_2 hydratase activity nor the acetate esterase activity was affected. When the modification was performed with a significant excess of phenylglyoxal, CO_2 hydratase and acetate esterase activities were diminished as well. That loss of activity was accompanied by the incorporation of an additional half dozen phenylglyoxals and, presumably, the modification of an equal number of arginine residues. The data in their entirety are interpreted to show (a) that the *p*-nitrophenylphosphatase activity is a unique property of carbonic anhydrase III and (b) that excessive amounts of the arginine-modifying reagent lead to unspecific structural changes of the enzyme as a result of which *all* of its enzymatic activities are inactivated.

Begining with the serendipitous discovery in our laboratory (Koester et al., 1977a,b) and with similar findings by Holmes (1977) and by Tashian's group (Tashian et al., 1978), mammalian muscle carbonic anhydrase is now accepted as a new enzyme species (CA III) that is different and distinguishable from the extensively researched carbonic anhydrases I and II originally found in erythrocytes. In fact, many of the research groups recognized for their work on carbonic anhydrases I and II have now also turned to studies on this third carbonic anhydrase as exemplified by the topics of a recent conference on the biology and chemistry of the carbonic anhydrases sponsored by the New York Academy of Sciences (Chegwidden et al., 1984).

Prior to our identification as carbonic anhydrase III (Koester et al., 1977a,b; Register et al., 1978) of what was then "basic muscle protein", we conducted numerous experiments designed to screen for a biological function of the unknown protein which at that time had been characterized only by its chemical and physical properties. Only the testing for acid phosphatase activity with the substrate *p*-nitrophenyl phosphate gave positive results, so thought was given initially to phosphatase activity being *the* primary function of basic muscle protein. Information on the phosphatase activity and its characteristics vs. those of the classical CO_2 hydratase and acetate esterase activities has since been reported (Koester, 1979; Koester et al., 1981; Pullan & Noltmann, 1984).

The possible location of a phosphatase catalytic site on muscle carbonic anhydrase suggested chemical modification of the enzyme by reagents known to interact with arginine residues, the potential phosphate binding sites. This approach would be especially valuable if it should prove possible to abolish the phosphatase activity without interfering with either the CO_2 hydratase or the acetate esterase functions. The present paper demonstrates that we were indeed successful in

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