Utilization of the free energy of the reversible binding of protein and modifying agent towards the rate-enhancement of protein covalent modification

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An analysis is presented of the catalytic factors responsible for the rate-enhancement that may be observed when a protein modification reaction is compared with a reaction of the same modifying agent with a model micromolecular compound exhibiting the same reactive group as the protein under study. It is seen that affinity-mediated rate-enhancement of protein modification is realized by the loss of activation entropy. On the assumption that attainment of maximal affinity-mediated rate-enhancement presents with an activation entropy of the protein modification reaction equal to zero, whereas the activation enthalpy of the reaction remains unchanged, it is shown that the value for maximal affinity-mediated rate-enhancement is equal to $e^{-\Delta S^\ddagger/R}$. Accordingly, protein modification reactions may be differentiated into (i) reactions the rate-enhancement of which (relative to the reaction of the same modifying agent with a model compound) is primarily entropy-controlled and (ii) reactions the rate-enhancement of which is primarily enthalpy-controlled. It is seen that modifying agents of low reactivity towards model compounds, but with a high, i.e. highly negative, activation entropy are better suited as prospective affinity-based protein-modifying agents, since the potential affinity-mediated rate-enhancement, and hence the selectivity, of these compounds is necessarily high. Kinetic and thermodynamic constants of the reaction of modifying agents with proteins, and with model compounds, and values of maximal affinity-mediated rate-enhancement, based on published data of the reaction of several modifying agents with model compounds, are presented and discussed.

INTRODUCTION

Protein modification reactions may exhibit an enhancement of rate when compared with the reaction of the same modifying agent with a micromolecular model compound possessing the same modifiable group as the protein under study. The question of the maximum extent of rate-enhancement of protein modification may be answered by the acceptance of certain formalistic assumptions, namely the description of the protein modification reaction by a single exponential function of reaction time, and also of an acceptable rate of dilution-induced enzyme re-activation (Rakitzis, 1989, 1990). In the present paper it is shown that the maximum extent of affinity-mediated rate-enhancement of protein modification may be determined if the entropy of activation of the reaction of the modifying agent under study with a model micromolecular compound is known.

The rate-enhancement frequently observed in cases of protein modification may be due to an array of factors obtaining in enzyme catalysis such as 'proximity effects, general acid or base catalysis, use of alternate pathways of covalent intermediates, orientation, strain and microenvironment, ion-pair and solvent effects' (Lennette & Plapp, 1979b). However, since rate-acceleration is accompanied by a lower free energy of activation ($\Delta G^\ddagger$) of the protein modification reaction, when this is compared with a model-compound modification reaction, it is of interest to inquire whether a lower $\Delta G^\ddagger$ value is due to a lower enthalpy of activation value ($\Delta H^\ddagger$) or a lower entropy of activation value ($\Delta S^\ddagger$) or both. (Throughout this paper, what is actually meant by 'entropy of activation' is 'entropy of activation with a negative sign, $-\Delta S^\ddagger$ '. Similarly, by the expression 'a decrease in the entropy of activation' is meant a decrease in the absolute magnitude of $\Delta S^\ddagger$. Thus a change of $\Delta S^\ddagger$ from $-50 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ to $-10 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ is referred to as a decrease in the entropy of activation.) This is of particular importance because the two thermodynamic magnitudes relate to different parts of the catalytic process. $\Delta H^\ddagger$ is related to the strength of the bonds to

be broken or formed during the reaction; thus, bending, distortion and polarization of bonds would be reflected in $\Delta H^\ddagger$, which is a measure of hyperreactivity or intrinsic reactivity of functional groups. On the other hand, $\Delta S^\ddagger$ is a measure of the randomness of the system and includes such effects as approximation of reactants, orientation, ion-pair formation, solvent effects, and stabilization of charge separation in the transition state' (Lennette & Plapp, 1979b). Again, in distinction to enzyme catalytic action, where the uncatalysed reaction may be studied, if at all, with great difficulty, protein covalent modification affords a ready means of ascertaining the contribution of changes in enthalpy as well as in the entropy of activation of the modification reaction, since in many recorded cases reaction of the modifying agent under study with a model micromolecular compound is fast enough to be conveniently studied.

Rate-enhancement of protein modification is thought to be due, at least in part, to the formation of a reversible protein-modifying agent adsorptive complex (affinity labelling). However, as Wolfenden & Frick (1987) point out: 'Adsorption of a substrate by a catalyst is not equivalent to chemical activation. Indeed, the adsorption process results in stabilization of the substrate, as indicated by the free energy released when binding occurs'. Again, as Jencks (1975) points out: 'Entropy is a measure of probability and the enzyme can decrease the amount of entropy that need be lost in a reaction by using binding forces to specific substrates to bring them together so that the reaction involves two adjacent molecules at the active-site rather than two separate, randomly oriented molecules in dilute solution. Thus, an enzyme may be regarded as an entropy sink or possibly, since it temporarily brings about a microscopic state of high order at no net cost in free energy, as an "entropic demon".... The utilization of entropy as a measure of the probability of reactions makes possible an estimation of the magnitude of the rate increase that may be brought about by increasing this probability, and also establishes an upper limit to the increase that may be assigned to this effect'. As in the case

of enzyme catalytic function, formation of a reversible protein-modifying agent complex may help towards an acceleration of reaction rate, since collection of the reactants on the protein surface removes the translational degrees of freedom of the reactants and contributes towards a decrease in the energy of activation of the protein modification reaction (affinity labelling) (Gutfreund & Knowles, 1967; Jencks, 1975; Fersht, 1977; Kluger, 1984). Consequently, a reaction in which the maximum effect of affinity-mediated rate-enhancement is observed is a reaction the activation entropy of which is zero. Contrariwise, reactions in which rate-enhancement is due to the use of alternative reaction pathways will present with low $\Delta H^\ddagger$ when compared with the model-compound reactions, while $\Delta S^\ddagger$ may remain unchanged. An altered $\Delta H^\ddagger$ value may also be due to the same basic mechanism of the model-compound modification reaction when this is compared with the protein modification event, in which, however, the rate-limiting step of the overall process has been changed (Bruice & Benkovic, 1963). Naturally, the 'par excellence' class of compounds that exhibit a different reaction mechanism is the class of enzyme-activated irreversible inhibitors (k_{cat} inhibitors, suicide substrates). It would appear, however, that extensive studies of this class of reactions have not included analysis of the thermodynamic parameters involved (Seiler *et al.*, 1978; Sandler & Smith, 1989).

THEORY

According to the theory of absolute reaction rates, the rate constant, k , of a reaction is given by the relationship:

$$k = e^m \cdot \frac{\kappa T}{h(c^0)^{m-1}} \cdot e^{\Delta S^\ddagger/R} \cdot e^{-\Delta H^\ddagger/RT} \quad (1)$$

where κ is the Boltzmann constant, h is the Planck constant, c^0 is the standard-state concentration, $1-m$ is the change in the number of molecules upon forming the activated complex, R is the gas constant and T is the absolute temperature (Wilkinson, 1980; Alberty, 1983). For a bimolecular reaction, the value of the pre-exponential factor of eqn. (1), at 25 °C, is equal to $1.69 \cdot 10^{13} \text{ M}^{-1} \cdot \text{s}^{-1}$ (Frost & Pearson, 1961; Wilkinson, 1980; Alberty, 1983). In the case of a protein modification reaction where affinity-mediated rate-enhancement is at a maximum, $\Delta S^\ddagger$ is equal to zero, whereas $\Delta H^\ddagger$ may be assumed to remain unchanged. Accordingly, the extent of maximal potential affinity-mediated rate-enhancement of protein modification is $e^{-\Delta S^\ddagger/R}$.

It would be of interest to see whether the action of protein-modifying agents is primarily affinity-mediated or is based on a change in reaction mechanism. Clearly, in the case of affinity-mediated rate-enhancement, the enthalpy of activation values of the protein modification reaction and of the reaction of the same modifying agent with a micromolecular model compound are identical, whereas the entropy of activation of the protein modification reaction is lower than the entropy of activation of the model-compound reaction. In fact, affinity-mediated rate-enhancement is entirely due to a decrease in the entropy of activation of the protein modification reaction, when this is compared with the model-compound modification reaction. However, although the case where the enthalpies of activation of the two reactions under comparison are widely different is one with a mechanism-based rate-enhancement factor, the contribution of the loss of activation entropy towards rate-enhancement may still be significant.

Determination of the thermodynamic activation parameters, i.e. $\Delta H^\ddagger$ and $\Delta S^\ddagger$, of the reaction of protein-modifying agents of high specificity with model compounds may be hampered by the low reactivity of such agents with model compounds, as is the case with substrate-derived diazomethyl ketones. These agents

fail to react with low-molecular-mass thiols (Leary *et al.*, 1977), and Brocklehurst & Malthouse (1978) suggest that the inactivation of papain by diazomethyl ketones is due to 'neighbouring group participation via transient thiohemiketal formation, rate-determining protonation by imidazolium ion and alkylation on sulphur via a three-membered cyclic transition state'. It thus becomes apparent that substrate-derived diazomethyl ketones, originally presented as 'par excellence' affinity labels, actually are mechanism-based irreversible enzyme inhibitors, albeit the contribution of affinity-mediated utilization of free energy is probably a significant part of this mechanism.

RELEVANCE TO EXPERIMENTAL DATA AND DISCUSSION

The thermodynamic and kinetic parameters of several cases recorded in the literature of the reaction of modifying agents with model compounds and with proteins are given in Table 1. It will be noted from Table 1 that, in the reaction of ethylmaleimide with GSH and with deoxyhaemoglobin, and in the reaction of methyl acetimidate with water and with aldolase, the $\Delta H^\ddagger$ value of the model compound reaction and that of the protein modification reaction are identical, indicating that rate-enhancement of protein modification by these compounds is affinity-mediated (it is of interest that the reaction of deoxyhaemoglobin with ethylmaleimide presents with an activation entropy value that actually is higher than that for the modification of GSH by the same compound, and is thus a case where protein modification is slower than the model-compound reaction). Also, in the case of the carbamoylation and of the methanesulphonylation of acetylcholinesterase, rate-enhancement by low-molecular-mass ligands is effected by a decrease in activation entropy, i.e. a less negative $\Delta S^\ddagger$ value, and is thus, in a sense, affinity-mediated. Finally, with such compounds as methyl iodide, iodoacetamide and bromovaleramide reaction with thiosubtilisin exhibits a significant difference in the value of $\Delta H^\ddagger$, indicating that the reactions under comparison are, in each instance, accomplished by different mechanisms. The reaction of methyl acetimidate with aldolase exhibits an entropy of activation value that is lower, i.e. more positive, than the entropy of activation of the methyl acetimidate hydrolysis reaction by a value of $-36.2 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. Since the entropy of activation of the latter reaction is $-81.6 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$, it is concluded that the fraction of protein-modifying agent binding energy utilized towards protein modification rate-enhancement is 0.40.

Determination of the extent of maximal potential affinity-mediated rate-enhancement of protein modification for several modifying agents is given in Table 1. It will be noted that modifying agents with a $\Delta S^\ddagger$ value close to zero, as is the case with methyl iodide and iodoacetamide, are poorly suited as candidates for effective protein labelling, unless the protein modification reaction in question exhibits a low $\Delta H^\ddagger$ value, i.e. unless factors such as use of alternative pathways, covalent intermediates or general acid or base catalysis are brought into play. It should also be noted that bromoacetate and phenyl acetates exhibit some of the highest values of model compounds for affinity-mediated rate-enhancement of protein modification on record. Interestingly, nitro group substitution in the *meta* or the *para* position of the ring brings about a decrease (i.e. a more positive value) in $\Delta S^\ddagger$ along with an increase in reactivity towards trimethylamine. It may accordingly be seen that ring substitutions that bring about an enhancement of reactivity may nevertheless also produce a decrease in affinity-mediated utilization of binding energy towards rate-enhancement of protein modification. Clearly, modifying agents exhibiting low reactivity towards micromolecular model compounds, but with a high, i.e. highly

Table 1. Rate-enhancement factors and thermodynamic constants of protein modification or of modification-induced enzyme inactivation reactions

Modifying agent	Protein or model compound	$\Delta H^\ddagger$ (kJ·mol ⁻¹)	$-\Delta S^\ddagger$ (J·K ⁻¹ ·mol ⁻¹)	k (m ⁻¹ ·s ⁻¹)	Rate-enhancement	Potential maximum affinity-mediated rate-enhancement	References
Phenyl acetates: R-C ₆ H ₄ OCOCH ₃ : R = <i>p</i> -NO ₂ R = <i>m</i> -NO ₂ R = <i>p</i> -Cl R = H	Trimethylamine Trimethylamine Trimethylamine Trimethylamine	51.5 50.6 52.3 54.0	88.4 112.3 127.8 132.0	71.5 5.7 0.513 0.133		41 100 734 800 4 740 000 7 856 000	Bruice & Benkovic (1963)
<i>p</i> -Nitrophenyl acetate	BSA			3 × 10 ⁴	420		Means & Bender (1975)
Ethylmaleimide	GSH	39.8	58.6	630		1136	Guidotti (1967)
Ethylmaleimide	Deoxyhaemoglobin	40.6	130.0	0.067	1.06 × 10 ⁻⁴		Whitaker & Lee (1972)
Chloroacetamide	Meraptoethanol	58.3	67.9	0.163		3480	Pavlic (1973); Pavlic & Wilson (1978)
Chloroacetamide	Ficin	14.6	174.0	13.3	81.5		
Methanesulphonyl fluoride	Acetylcholinesterase	58.2	66.1	3.1		2806	
Methanesulphonyl fluoride + tetraethylammonium	Acetylcholinesterase	50.6	11.3	7.44	2.4*		
<i>o</i> -Nitrophenyl dimethylcarbamate	Acetylcholinesterase	53.5	107.1	76.5		393 100	Roufogalis & Wickson (1974)
<i>o</i> -Nitrophenyl dimethylcarbamate + CaCl ₂	Acetylcholinesterase	57.7	92.9	84.1	1.1*		
<i>o</i> -Nitrophenyl dimethylcarbamate + gallamine	Acetylcholinesterase	59.0	83.3	176.0	2.3*		
Methyl iodide	GSH	72.1	3.9	0.919		2	Halász & Polgár (1976)
Methyl iodide	Thiosubtilisin	50.0	65.4	4.15	4.5		
Iodoacetamide	GSH	51.9	43.2	27.2		181	
Iodoacetamide	Thiosubtilisin	62.0	21.7	6.33	0.24		
Bromovaleramide	GSH	63.7	54.8	0.06		729	
Bromovaleramide	Thiosubtilisin	50.4	80.4	0.591	10.0		
Aryl isothiocyanates: R-C ₆ H ₄ -NCS: R = 4-Cl R = 4-I R = 4-CH ₂ CO ₂ H R = 4-CH ₂ CO R = 4-Br R = H	Thioglycollate Thioglycollate Thioglycollate Thioglycollate Thioglycollate Thioglycollate	29.9 -8.83 37.1 61.0 55.2 44.8	66.1 110.9 30.0 32.2 36.8 48.1	3.1 2.62 0.317 3.50 1.93 1.05		2837 621 000 37 48 84 326	Drobnica & Augustin (1965)
Bromoacetate	Histidine hydantoin	69.5	98.3	2.0 × 10 ⁻³		1.6 × 10 ¹⁰	Lennette & Plapp (1979a,b)
Bromoacetate	RNAase	46.0	112	2.1 × 10 ⁻²	1050		
Methyl acetimidate	Water	58.0	81.6	1.095 × 10 ⁻³ †	2.95 × 10 ⁸		Makoff & Malcolm (1981)
Methyl acetimidate	Aldolase	58.0	45.4	0.0636	5800		

* Rate-enhancement considered versus modification rate of the ligand-free enzyme.

† Corrected for the participation of water by dividing by 55.5 M.

negative, activation entropy, are better suited as prospective affinity-based protein-modifying agents, since the rate-enhancement, and hence the selectivity of labelling, to be obtained with these agents will be quite high.

High rate-enhancement factors when protein modification reactions are compared with model-compound reactions have been observed in the case of the alkylation of creatine phosphokinase by iodoacetamide (65-fold rate-enhancement; Jones *et al.*, 1975), the alkylation of RNAase by bromoacetamidopyrimidine nucleosides (9100-fold and 400-fold rate-enhancement; Hummel *et al.*, 1987) and the irreversible inhibition of sulphate transport in human erythrocytes by an isothiocyanic acid ester (1045-fold rate enhancement; Rakitzis, 1987). However, it is not clear whether, in these cases, rate-enhancement of protein modification is due to the utilization of binding energy or to the use of a different reaction pathway. From the data presented in Table 1 it may be surmised that reactions where rate-enhancement of protein modification is due to a decrease in activation entropy alone are in the minority. Clearly, determination of the thermodynamic parameters of the reaction of a modifying agent with a micromolecular model compound may be of value in indicating the strategy to be followed (affinity labelling versus mechanism-based rate-enhancement) in the development of highly specific protein-modifying agents.

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