much less stable in both cases (Wiseman et al., 1975). Reduced forms contain FeII, which is a high-spin-state form, and this may explain the lower stability, although there is a possibility of a conformational difference for the FeII form associated with its mechanistic role in oxygen binding. This is another possible case, therefore, of a mechanistic advantage and stability being mutually exclusive.

It is clear that, for a cytochrome P-450 acting on a Type I substrate, the enzyme will be more unstable in use (i.e. in the presence of substrate if no compensating stabilization occurs on its binding) than in storage, because the substrate converts the enzyme into the high-spin form. Type II substrates, however, have lower rates, but higher stability in use. Several Type II ligands, including enzyme inhibitors, should be tested as stabilizers for storage of these enzymes.

Consideration of enzyme mechanism may therefore be of general interest in relation to stability. Induced fit and other likely mechanisms of increasing rates of enzyme reactions may also diminish the stability of the enzyme. Therefore, although the efficiency of the enzyme, expressed as $k_{cat}/K_m$, may be higher, lowered stability may also result in some cases (especially in highly evolved enzymes) from adverse conformational or electronic changes. These factors, where opposing, must be considered in selecting enzymes—and in the future in designing them!


Approaches to enzyme stabilization

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One of the major factors severely limiting practical applications of enzymes is their relative instability under operational conditions. By far the most important mode of enzyme inactivation is thermal (Klibanov, 1982). Other factors that frequently bring about inactivation of enzymes are oxygen, organic solvents, $\text{H}_2\text{O}_2$, extreme pH etc. (Putman 1954; Joly, 1965).

Despite some recent advances in the area of enzyme stabilization (Wiseman, 1978; Klibanov, 1979; Schmid, 1979), many serious problems remain. This has stemmed from the fact that in most cases complete mechanisms of enzyme inactivation are still obscure. However, in the present paper I attempt to demonstrate that often even rather general information concerning the pathways (or their fragments) of enzyme inactivation can lead to development of rational approaches to stabilization of enzymes provided that one properly employs this information along with the common sense.

Stabilization against thermoninactivation

On the basis of the data available from the literature, the scheme of irreversible thermal inactivation of enzymes shown in Scheme 1 can be put forward. The first and crucial step in enzyme thermoninactivation is partial unfolding of the protein molecule (Kauzmann, 1959; Tanford, 1968). The subsequent events very much depend on the enzyme, but generally can be divided into two groups: (i) covalent and (ii) non-covalent:

(i) This group comprises very thermostable enzymes, e.g. egg-white lysozyme and bovine pancreatic ribonuclease, which require prolonged boiling for considerable inactivation. Covalent changes occurring in enzymes at such drastic conditions include hydrolysis of peptide and amide bonds, racemization of amino acids, hydrolytic scission of disulphides, $\beta$-elimination etc. (Feeney, 1980).

(ii) Apparently most of enzymes belong to this category. First, unfolded molecules can interact with each other, resulting in their aggregation and, in turn, loss of solubility and catalytic activity. Secondly, unfolded molecules can intramolecularly refold into new structures, different from the native enzyme conformations, to form kinetically or thermodynamically stable structures that are catalytically inactive (Klibanov & Mozhaev, 1978).

Even the simplified scheme of enzyme thermal inactivation presented in Scheme 1 affords some rationales for enzyme stabilization. For example, the obvious way to decelerate thermoninactivation seems to be to prevent or diminish protein molecule unfolding. This has been achieved by multi-point attachment (either covalent (Martinek et al., 1977a) or non-covalent (Martinek et al., 1977b)) of enzymes to solid supports or by intramolecular cross-linking of enzyme molecules (Zaborsky, 1974; Torchilin et al., 1978).

If thermoninactivation occurs as a result of aggregation, this process can be stopped by enzyme immobilization, since the latter results in mutual spatial fixation of enzyme molecules with a consequent loss in ability for intermolecular interactions.

The situation is not hopeless even after an enzyme has been thermoninactivated, provided that this inactivation is non-covalent. Such 'irreversible' thermal inactivation can be reversed by unfolding the inactivated enzyme in concentrated solutions of guanidinium chloride or urea (with or without concurrent reduction of disulphide bonds), followed by removal of the denaturant and refolding of the enzyme. This procedure has been successfully used for reactivation of both thermooaggregated enzymes (Westhead, 1964; Davies & Abraham, 1974; Tokushige & Eguchi, 1978; Little & Johansen, 1979) and the enzymes intramolecularly folded into incorrect structures (Klibanov & Mozhaev, 1978; Martinek et al., 1980).

Stabilization against oxygen inactivation

Mechanisms of inactivation of enzymes by $\text{O}_2$ are absolutely different from those of thermoninactivation. Owing to the very nature of this type of enzyme inactivation, it involves oxidation of protein functional groups either by $\text{O}_2$ itself or by such secondary reactive species as $\text{OH}^\cdot$, $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2^\cdot$.

Oxygen inactivation of enzymes and stabilization thereof can be illustrated by our studies with Clostridium pasteurianum.
The scheme represents the events during thermal inactivation of enzymes. It shows the transition from a native catalytically active enzyme through unfolding, refolding, and ultimately aggregation or incorrectly folded enzyme.

### Practical approaches to chemical modification of enzymes for industrial use

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During immobilization of enzymes, secondary effects are often seen, e.g., apparent increase in stability, resistance to proteolytic degradation and elimination of allergenic properties. These can in most cases be described as steric or diffusional effects due to the matrix of the immobilized enzyme. Nevertheless, such effects are not necessarily related to immobilization. In the biochemical literature there are many examples of a change in the properties of soluble enzymes as a result of chemical modification (Means & Feeney, 1971). These changes can be caused by steric effects, but more often by chemical effects, such as hydrophobic or hydrophilic interactions. The industrial aspects of chemical

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**References**