butyl ester), was done in the conventional way. Using this combination, [D-Ala$^2$-D]DHI was obtained in crystalline form and gave about 50% of the insulin activity in the mouse-convulsion assay.

Insulins from different species are analogues formed in the course of evolution and are very useful in structure-and-function studies. We have worked out a simple method for isolating these analogues. Insulins of chicken, snake (Cao et al., 1980) and goose (Xu et al., 1982) have been isolated in crystalline form. The crystalline shape of snake insulin is very unusual, it is dodecahedral instead of rhombohedral. Snake $\beta$-granules were studied by Watari & Hotta (1976), by high-voltage electron microscopy, and by Lange (1980), by electron microscopy and electron diffraction. They also found dodecahedral microcrystals and two possible space groups; P4$_3$21 and P2$_1$2$_1$2 were established. The crystal morphology of the insulin activity in the mouse-convulsion assay.

Although the determination and refinement of the crystal structure of insulin by X-ray-diffraction methods was begun in 1967 by the Peking Insulin Structure Research Group. In 1970, and later in 1971, results at 0.4 and 0.25 nm (4.0Å and 2.5Å) resolution respectively were obtained. Further work, extending the resolution to 0.18 nm (1.8Å), was completed in 1973 (Peking Insulin Structure Research Group, 1974). In 1979 a refined model for the insulin structure at 1.8Å resolution with an R-factor (R = $\sum |F_o|^2 - |F_c|^2/|F_o|^2$) of 0.21 was obtained (Wang et al., 1982a,b). The refinement of the insulin structure at 0.12 nm (1.2Å) resolution is now in progress.

Some problems in relation to the structures of insulin derivatives

LIANG DONG-CAI, DAI JIN-BI, DAVID STUART, WAN ZHU-LI, ROSEMARY TODD, YOU JUN-MING and LOU MEI-ZHEN

Institute of Biophysics, Academia Sinica, Beijing, China

After our scientific workers first succeeded in the total synthesis of bovine insulin with full biological activity by chemical methods, the analysis of the crystal structure of insulin by X-ray-diffraction methods was begun in 1967 by the Peking Insulin Structure Research Group. In 1970, and later in 1971, results at 0.4 and 0.25 nm (4.0Å and 2.5Å) resolution respectively were obtained. Further work, extending the resolution to 0.18 nm (1.8Å), was completed in 1973 (Peking Insulin Structure Research Group, 1974). In 1979 a refined model for the insulin structure at 1.8Å resolution with an R-factor (R = $\sum |F_o|^2 - |F_c|^2/|F_o|^2$) of 0.21 was obtained (Wang et al., 1982a,b). The refinement of the insulin structure at 0.12 nm (1.2Å) resolution is now in progress.

Although the determination and refinement of the crystal structure of insulin has given us very important structural information on the relationship between the structure and function of insulin, it is still unable to elucidate the mechanism of insulin action. Therefore the determination of the three-dimensional structures of a series of insulin analogues and the analysis of the fine differences in their conformations will undoubtedly be significant for studying the structure-function relationship of insulin.

X-ray-crystallographic studies on some insulin analogues modified at the N-terminus of the B-chain

The N-terminus of the B-chain is one of the important regions for studying the structure-function relationship of insulin. For a long time, it was considered that the B-chain N-terminus was mainly important for immunological reactivity and not so important for biological activity. The studies on des-(B1-Phe)-insulin, which retains almost complete biological activity as compared with insulin, supported this point of view (Brandenburg, 1969). However, extensive investigations on this region (Saunders et al., 1977) have shown that the B-chain N-terminal residues may in some way be involved in the interaction of insulin with its receptor. Therefore studies on the three-
dimensional structures of B-chain N-terminal modified insulin analogues have been undertaken with the aim of assessing the functional importance of this region in contributing, directly or indirectly, to the biological activity of insulin. During the past two years our efforts have been concentrated on growing crystals of some of the analogues and on preliminary X-ray crystallographic studies.

Purified des-(B1-2)-insulin (hereafter insulin will be shortened to Ins) and des-(B1-3)-Ins were prepared in our department by Dr. Lei and his co-workers, and (l-Met)-B0-Ins and (l-Arg)-B0-Ins were kindly sent to us by Professor C. C. Yip from Toronto University. Good single crystals of these analogues were obtained from citrate buffer containing about 0.08% ZnCl₂ and 30% acetone, conditions quite similar to those used for growing rhombohedral pig 2Zn-insulin crystals. Fig. 1 shows the crystals of des-(B1-2)-Ins, des-(B1-3)-Ins, (l-Met)-B0-Ins and (l-Arg)-B0-Ins. All of these are suitable for X-ray analysis.

Although the conditions under which the crystals were grown were in each case very close to those for pig 2Zn-insulin, des-(B1-2)-Ins and (l-Met)-B0-Ins gave orthorhombic do-decahedral crystals of a cubic space group, while the crystals of (l-Arg)-B0-Ins and des-(B1-3)-Ins were isomorphous with the rhombohedral pig 2Zn-insulin, as may be seen from Fig. 1. The crystallographic parameters for des-(B1-2)-Ins, (l-Met)-B0-Ins and (l-Arg)-B0-Ins were determined from precession photographs, and the specific weights of these crystals were obtained by the isopycnic method. The results are listed in Table 1.

Note that, under similar conditions, (l-Arg)-B0-Ins crystals have a rhombohedral unit cell, while (l-Met)-B0-Ins and des-(B1-2)-Ins crystallize in the space groups P2₁,₃ and P4₃,2 respectively, which differ from both the P4₃,2 snake-insulin crystals and the I₂,₃ cubic porcine-insulin crystals. Presumably these differences in packing of the hexamers indicate that the replacement of the N-terminal region of the B-chain alters in

<table>
<thead>
<tr>
<th>Species</th>
<th>(l-Met)-B0-Ins</th>
<th>(l-Arg)-B0-Ins</th>
<th>Des-(Phe-Val)-B1-2-Ins</th>
</tr>
</thead>
<tbody>
<tr>
<td>a (Å)</td>
<td>73.50</td>
<td>81.82</td>
<td>97.43</td>
</tr>
<tr>
<td>b (Å)</td>
<td>73.50</td>
<td>81.82</td>
<td>97.43</td>
</tr>
<tr>
<td>c (Å)</td>
<td>90</td>
<td>35.05</td>
<td>90</td>
</tr>
<tr>
<td>α (°)</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>β (°)</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>γ (°)</td>
<td>90</td>
<td>120</td>
<td>90</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Cubic</td>
<td>Rhombohedral</td>
<td>Cubic</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁,₃</td>
<td>R3</td>
<td>P4₃,2</td>
</tr>
<tr>
<td>Crystal density (D₀) (g/cm³)</td>
<td>1.15</td>
<td>1.20</td>
<td>1.21</td>
</tr>
<tr>
<td>Asymmetric unit molecule</td>
<td>22.00</td>
<td>203.206</td>
<td>924.854</td>
</tr>
<tr>
<td>Unit cell volume (Å³)</td>
<td>397065</td>
<td>28</td>
<td>61</td>
</tr>
</tbody>
</table>

* Note 10 Å = 1 nm (the S.I. unit).
some way the conformation of the insulin structure and/or the distribution of the charges on the surface of the insulin molecule, and so on the surface of its hexamer.

In rhombohedral 2Zn-insulin crystals, the hexamer is approximately an ellipsoid of some 5 x 3 nm (50 Å x 30 Å) and the distance between hexamer centres is about 5 nm (50 Å). Let us consider the packing of (L-Met)-B0-Ins hexamers in the crystal. There are 12 general positions in the space group P2_13, four hexamers per unit cell, and therefore a single dimer in an asymmetric unit. This means that the 3-fold axis of the hexamer must lie on a crystallographic 3-fold axis and thus the hexamer centre can be placed at positions x, x, x, etc., as shown in Fig. 2.

In this case the orientation of the two-fold axis relating the dimers of each hexamer is undefined. If the fractional coordinate of the centre of the hexamer (x) is taken to be 1/4 or 3/4, the packing of the hexamers is very reasonable and the distance between their centres is about 5.2 nm (52 Å), some 0.2 nm (2 Å) larger than that in the 2Zn-insulin rhombohedral crystals.

A similar analysis suggests a reasonable packing of des-(B1-2)-Ins molecules in their P4_32 unit cell, as presented in Fig. 3. In this case there are eight hexamers in a unit cell in special positions on the 3-fold axes with co-ordinates x, x, x etc. In this case if x is 1/4 or 3/4, the distance between hexamers is some 4.9 nm (49 Å), indicating a rather closer packing after the removal of B1-Phe and B2-Val. As above, the orientation of the dimer of des-(B1-2)-Ins is arbitrary.

These possible packings of the (L-Met)-B0-Ins and des-(B1-2)-Ins molecules need confirmation by further structural analysis and to this end three-dimensional structural studies on (L-Met)-B0-Ins are now in progress in our laboratory.

Some results of X-ray-crystallographic studies on snake insulin

The primary structure of insulin from the snake *Zaocys dhumnades dhumnades* Cantor as recently reported (Zhang et al., 1981), differs from most other insulins by the presence of B5-Arg, B16-Phe, B18-Ile, B25-Tyr and B29-Arg; but differs from rattlesnake insulin only at A15-Glu and B30-Thr (Kimmel et al., 1976). X-ray-crystallographic studies on this snake insulin have been done in collaboration with Professor Y. S. Zhang and his co-worker Q. P. Cao of the Shanghai Institute of Biochemistry.

Single crystals suitable for X-ray analysis were obtained from a buffer solution consisting of 0.6% snake insulin, 0.5% citrate, 8% acetone and 0.07% zinc acetate. Both the orthorhombic dodecahedral shape and the X-ray diffraction indicated that these snake-insulin crystals possess cubic symmetry. The Laue symmetry and the systematic extinctions determine the space group unambiguously as P4_32, a = 0.6731 nm (67.31 Å). The specific weight of the crystals is 1.24; then from the unit cell volume and the molecular weight of snake insulin, their solvent content may be calculated as 38%. There are 24 insulin molecules per unit cell, thus each asymmetric unit contains one molecule. Crystal parameters are given in Table 2.

Although there are substantial differences between the primary structures of snake and pig insulin, residue B10, the histidine residue that co-ordinates the Zn^{2+} ions in the 2Zn-type pig hexamers, is conserved. Since the crystallization is done in the presence of Zn^{2+}, hexamers of snake insulin may well still be formed by co-ordinating with Zn^{2+} on the 3-fold axis, as in rhombohedral pig 2Zn-insulin. In space group P4_32 there are 24 general positions and two sets of four special positions, each with symmetry 32. The only reasonable packing of hexameric snake insulin molecules in space group P4_32 is in fact as shown in Fig. 4. If the orientation of the hexamers is not taken into

---

**Table 2. Snake-insulin crystal data**

<table>
<thead>
<tr>
<th>Crystal system</th>
<th>Cubic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit cell parameter</td>
<td>a = b = c = 67.31 Å</td>
</tr>
<tr>
<td></td>
<td>a = β = γ = 90°</td>
</tr>
<tr>
<td>Space group</td>
<td>P4_32</td>
</tr>
<tr>
<td>Crystal density</td>
<td>D_m = 1.24 g/cm^3</td>
</tr>
<tr>
<td>Asymmetric unit molecule</td>
<td>1</td>
</tr>
<tr>
<td>Unit cell volume</td>
<td>V = 304957 Å</td>
</tr>
<tr>
<td>Unit cell solvent</td>
<td>38%</td>
</tr>
</tbody>
</table>

* Note 10 Å = 1 nm.
account, the packing of snake-insulin hexamers in the P4,32 unit cell is the same as that of (L-Met)-BO-Ins hexamers in P2\textsubscript{3} (Fig. 2). However, each hexamer of snake insulin is limited by a 3-fold axis and also by three 2-fold axes. In 22z-insulin rhombohedral crystals, each dimer has a non-crystallographic 2-fold axis perpendicular to the crystallographic 3-fold axis relating the three dimers of each hexamer, and the same is presumably true in the P2\textsubscript{3} crystals of (L-Met)-BO-Ins. But for snake-insulin crystals, each asymmetric unit has only one molecule and the hexamers have proper 3 symmetry, with three 2-fold axes perpendicular to a 3-fold axis, thus fixing the position of each molecule as shown in Fig. 5. We hope to confirm this proposed packing by further structural analysis.

The three-dimensional structure of des-pentapeptide-(B26–30)-insulin

Studies on insulin analogues with amino acid residues removed from the C-terminus of the B-chain indicate that while des-pentapeptide-(B26–30)-insulin (DPI) retains almost the full biological activity of insulin (Insulin Research Group of Shanghai et al., 1976), des-hexapeptide-(B25–30)-insulin (DHI) has only 40% activity, and des-heptapeptide-(B24–30)-insulin (DHPI) completely loses activity in vivo. Thus we conclude that both B24- and B25-Phe are important for the biological activity of insulin. Furthermore, Dr. Tager and his co-workers discovered in 1979 (Tager et al., 1979) that an abnormal insulin isolated from the pancreas of a diabetic patient contains a non-crystallographic 2-fold leucine-for-phenylalanine substitution at B24 and B25 and has decreased biological activity. What is the function of these two residues? Do they form part of the active site of the insulin molecule? Or, alternatively, does the loss of them induce a conformational transition of the whole molecule, making it inactive? The analysis of the three-dimensional structures of the above three insulin analogues (DPI, DHI and DHPI) would give us some valuable information for elucidating the structure-function relationship of these two phenylalanine residues. Good hexamers have proper 32 symmetry, with three 2-fold axes relating the three dimers of each hexamer, and the same is presumably true in the P2\textsubscript{3} crystals of (L-Met)-BO-Ins. But for snake-insulin crystals, each asymmetric unit has only one molecule and the hexamers have proper 3 symmetry, with three 2-fold axes perpendicular to a 3-fold axis, thus fixing the position of each molecule as shown in Fig. 5. We hope to confirm this proposed packing by further structural analysis.

An anomalous difference Patterson was calculated using data to a spacing of 0.24 nm (2.4 Å), which had been carefully processed. From the Harker section, the position of a single Cd\textsuperscript{2+} ion was clearly visible in the anomalous difference Patterson. Thus, we conclude that there is only one molecule in an asymmetric unit and almost 70% of the crystal volume is occupied by protein molecules; this corresponds to a more compact arrangement than is usual in protein crystals (Matthews, 1977).

Two apparently satisfactory heavy-atom derivatives reported at low resolution (Wang et al., 1980; Li et al., 1981), have subsequently proved disappointing and we have been unable to extract any useful medium- or high-resolution phase information from either. An alternative approach to the isomorphous replacement method, was to attempt to solve the phase problem by using the so-called 'Resolved Anomalous Phasing' (RAP) method. This novel technique has been pioneered by Hendrickson (Hendrickson & Teeter, 1981), who brought together the heavy-atom method from small-molecule crystallography with his own experience of the anomalous scattering method applied to large molecules to provide, for protein crystals containing anomalously scattering atoms (e.g. Cd\textsuperscript{2+}), a method of solving the phase problem.

An anomalous difference Patterson was calculated using data to a spacing of 0.24 nm (2.4 Å), which had been carefully processed. From the Harker section, the position of a single Cd\textsuperscript{2+} ion was clearly visible in the anomalous difference Patterson. Thus, we conclude that there is only one molecule in an asymmetric unit and almost 70% of the crystal volume is occupied by protein molecules; this corresponds to a more compact arrangement than is usual in protein crystals (Matthews, 1977).

This result was corroborated by the use of the rotation function, and some 13 putative a-carbon positions were recorded in regions suggestive of helical structure. These co-ordinates were then used to guide the rotation and translation of the model of insulin (in the Beijing convention) on to DPI with the superposition algorithm of Hendrickson (1979). The resultant root-mean-square deviation between the measured and transformed co-ordinates was 0.099 nm (0.99 Å).

<table>
<thead>
<tr>
<th>Table 3. DPI crystal data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal system</td>
</tr>
<tr>
<td>Unit cell parameter</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Space group</td>
</tr>
<tr>
<td>Crystal density</td>
</tr>
<tr>
<td>Asymmetric unit molecule</td>
</tr>
<tr>
<td>Unit cell volume</td>
</tr>
<tr>
<td>Unit cell solvent</td>
</tr>
</tbody>
</table>

* Note 10 Å = 1 nm.
Temperature factors and 50 water molecules were included in the refinement. The final R-factor for all data from 0.1 nm (10 Å) to 0.24 nm (2.4 Å) was 0.182 and the root-mean-square deviation of covalent-bond lengths from their 'ideal' values was 0.0027 nm (0.027 Å). We estimate [both from the method of Luzatti (1952) and from the comparison of the DPI and insulin co-ordinates] that the average error for the well-determined atoms probably does not exceed 0.02 nm (0.2 Å). This model of DPI is displayed in Fig. 6.

DPI, like insulin, consists of an A-chain with two α-helices, one at each end of the chain, separated by a loop region, and a B-chain with an α-helix running from residue B9 to B19. The β-structure observed at the C-terminal end of the insulin B-chain cannot, of course exist, since each hydrogen bond involves one of the residues that is lost in DPI, and in fact, as expected from solution studies (Insulin Research Group of Shanghai, 1976), we observe (as can be seen in Fig. 6) that the DPI molecules do not form 2Zn-type hexamers or dimers. Further internal stability is

**Fig. 6.** The structure of DPI.

Symbols: ○, oxygen; ●, nitrogen; Cd, cadmium; S-S, disulphide bridge.

**Fig. 7.** The co-ordination of cadmium in the DPI crystals

Note that 10 Å = 1 nm (the SI unit).
Fig. 8. A comparison of the structures of DPI and molecule I of 2Zn-insulin
Symbols: ___, main chain of DPI; ---, main chain of molecule I of 2Zn-insulin; Cd, cadmium; Zn, zinc; S-S, disulphide bridge. Note that 10 Å = 1 nm.

conferred on the insulin molecule by the presence of two salt bridges; of these the one between the C-terminus of the A-chain and B22-Arg is still found in DPI, while the other (between the N-terminus of the A-chain and the carboxylate group of A4-Glu) is broken.

Although the DPI molecules do not aggregate, it is noteworthy that each molecule co-ordinates four Cd\(^{2+}\) ions with five of its polar groups (A15-Gln, B5-His, B10-His, B13-Glu and the C-terminus of the B-chain). In other words each Cd\(^{2+}\) ion is co-ordinated with five polar groups from four adjacent DPI molecules (Fig. 7), forming a close network of interactions between the DPI molecules in the crystal. This network probably helps to stabilize the close packing of the DPI molecules to produce a lattice with rather low solvent content and good crystalline order (the crystals diffract strongly to high resolution). Further investigation of the groups that co-ordinate the Cd\(^{2+}\) reveals that, with the exception (discussed below) of B25, the co-ordination has wrought rather little change in the backbone structure, perhaps hinting that the Cd\(^{2+}\) co-ordination, while important for crystallization, may not be related to the biological activity of DPI.

To perform a detailed, quantitative comparison of the structures of DPI and 2Zn-insulin, molecules I and II of insulin (co-ordinates derived from an R = 0.178 model at 0.16 nm (1.6 Å) resolution) were superimposed on the DPI molecule using the main chain and \(\alpha\)-carbon atoms of the 36 most similar residues. The root-mean-square deviation of the 36 \(\alpha\)-carbon atoms after superimposition, was 0.06 nm (0.6 Å) for molecule I, while the corresponding value for molecule II was 0.1 nm (1 Å). This confirms that the structure of molecule I in insulin probably represents the more 'typical' insulin structure (Cutfield et al., 1981). Fig. 8 shows the DPI/insulin molecule I comparison and clearly indicates the great degree of structural homology between this molecule and much of DPI. However there are some big differences between these two molecules, especially in the side chains, but also in some regions of the main chain. These latter changes are mainly at the N- and C-termini of the B-chain and to a lesser extent in the loop region of the A-chain (Fig. 9).

Fig. 9. A comparison of the conformation of residues B24 and B25 in DPI and molecule I of 2Zn-insulin (Ins on Figure)

The \(\alpha\)-helices of both the A- and B-chains superimpose quite well; however more detailed analysis reveals that there are some changes in their relative orientations. The distance between the position of Zn\(^{2+}\) in insulin and that of Cd\(^{2+}\) in DPI is 0.33 nm (3.3 Å); since in both molecules B10-His co-ordinates the metal...
ion, this displacement is accommodated in the DPI molecule by a corresponding movement of the imidazole ring.

As mentioned above, the two phenylalanine residues B24 and B25 are important for the biological activity of insulin. Fig. 9 shows the change in conformation of these two residues in DPI compared with insulin (molecule I). We can see that a large change in conformation occurs at B25, while B24 is in a very similar position in both molecules. From these results we would like to think that both the chemical properties and the precise conformation of B24 are important for biological activity, while perhaps the chemical properties of B25 are more important than its conformation. We hope that the important biological significance of the C-terminal region of the B-chain of insulin, particularly B24 and B25, will be further clarified by the solution of the DHPI and des-octapeptide-insulin structures.


A new fluorescent probe for the study of the allosteric properties of D-glyceraldehyde 3-phosphate dehydrogenase

C. L. TSOU, G. Q. XU, J. M. ZHOU and K. Y. ZHAO
Institute of Biophysics, Academia Sinica, Beijing, China

Although the four subunits that constitute the D-glyceraldehyde 3-phosphate dehydrogenase isolated from a number of different sources are identical in amino acid sequences (Davidson, 1967; Jones & Harris, 1972; Hocking & Harris, 1973), they behave differently in their NAD-binding properties as well as in reactions toward some chemical modification reagents. It has long been known that the muscle (Conway & Koshland, 1968; de Vijlder et al., 1969) and the Bacillus stearothermophilus (Allen & Harris, 1973) enzymes show different stoichiometry in NAD binding; the co-operativity of NAD binding with the yeast enzyme is either positive (Kirschner et al., 1966) or of a mixed type (Cook & Koshland, 1970), depending on experimental conditions. These have been explained either by induced sequential conformational changes of the subunits (Koshland et al., 1966; Henis & Levitski, 1980) or by a non-identity in conformation of the subunits pre-existing in the tetrameric molecule (MacQuarrie & Bernhard, 1971; Moras et al., 1975).

It has been shown in previous publications from our laboratory that when the active-site Cys-149 of the rabbit muscle (Ho et al., 1979) or the Bacillus stearothermophilus (Ho et al., 1980a) enzyme is carboxymethylated, u.v. irradiation in the presence of NAD+ leads to the formation of a fluorescent derivative with an emission maximum at 410 nm. A comparison of a number of chemical modification reagents has shown that it is necessary for the active-site Cys-149 to be modified with a reagent carrying a free carboxy group so as to allow the formation of this fluorescent derivative (Ho et al., 1980a). On the other hand, the presence of NAD+ or an NAD+ analogue with a substituted pyridine ring is also required (Xu & Tsou, 1982). These findings and the facts that this fluorescent derivative has fluorescence and u.v.-absorption properties (Ho et al., 1979; Ho & Tsou, 1979) closely resemble those of the KCN adduct of NAD+ strongly suggest that it is formed through covalent linkage of the carboxyalkyl group introduced at Cys-149 and the pyridine ring of NAD+ at the active site. Unlike the tightly bound NAD+ molecules of the holoenzyme from rabbit muscle, this fluorescent derivative of NAD+ cannot be removed from the enzyme protein by treatment with activated charcoal under similar conditions (Ho et al., 1979). Moreover, a circular-dichroism study of the rabbit muscle enzyme carrying the fluorophore (Lian & Tsou, 1981) has indicated that no gross conformational change has taken place accompanying the formation of this fluorescent derivative. This is also supported by the fact that it is easily crystallized under similar conditions and in the same crystal form as the native holoenzyme.

It is the purpose of the present paper to show that the stoichiometry of the photochemical reactions leading to the formation of this fluorescent derivative with the rabbit muscle and particularly with the yeast enzymes seems to suggest an inherent difference in conformation of the four subunits. Experimental details will be published elsewhere (Xu & Tsou, 1983; Zhou et al., 1983).

(1) The formation of the fluorophore is a 'half-of-the-sites' reaction for the rabbit muscle enzyme

The effect of NAD+ concentration on the formation of the fluorescent derivative is shown in Fig. 1. As is well known, this enzyme binds NAD+ with negative co-operativity, and the first two molecules of NAD+ are bound very tightly (Conway & Koshland, 1968). The carboxymethylated of the active-site Cys-149 residue somewhat weakens the NAD+ binding, but does not greatly affect the negative co-operativity (Zhao et al., 1980). Fig. 1 shows that the amount of fluorescent derivative formed increases linearly with the amount of NAD+ present up to an NAD+/enzyme ratio of 2, again showing tight binding for the first two NAD+ molecules. Extrapolation of this initial linear portion of the curve intercepts the extrapolation of the maximal